# Heterogeneity of VanA phenotype glycopeptide-resistant enterococci in Scotland

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# Abstract

Although traditionally perceived as being of little clinical significance, enterococci have emerged as major nosocomial pathogens, primarily due to the emergence of multi-drug resistant strains. In cases of serious enterococcal infection, the optimal therapeutic option is the synergistic combination of a cell wall-active agent with an aminoglycoside. By the late 1980s, high-level resistance to both penicillin and the aminoglycosides was increasing in prevalence amongst clinical enterococcal isolates, thus compromising this synergistic combination. It was the emergence of glycopeptide resistance in the late 1980s, however, that truly rendered some strains untreatable by proven therapeutic agents.

There are currently five distinct phenotypes of glycopeptide resistance in enterococci, the most prominent being VanA, which is characterized by high-level, inducible resistance to vancomycin and teicoplanin, and is conferred by the Tn*3*-related transposon, Tn*1546*. Whilst glycopeptide-resistant enterococci (GRE) are a significant nosocomial pathogen, their epidemiology is extremely complex and is not restricted to the hospital setting. In Europe, GRE can be readily isolated from a variety of environmental and animal sources, as well as from healthy non-hospitalized individuals.

The aim of this study was to investigate the epidemiology of VanA GRE in Scotland, and to determine the degree of diversity amongst the Tn*1546*-related elements conferring resistance. GRE were collected over a five-year period from eight hospitals across Scotland. Fifty-five GRE isolates were collected in total, 48 of which were found to be *vanA*-positive. In addition, a five-month survey of enterococcal epidemiology was performed, during which time 94 enterococcal isolates were collected, two of which were found to be *vanA*-positive.

All isolates were examined by PFGE analysis. Twenty-six of the 55 nosocomial GRE isolates collected during the five-year study period were isolated during the course of an outbreak of GRE infection in the renal unit of the Royal Infirmary of Edinburgh in 1995. Many isolates arising from the outbreak were clonal in nature, with an outbreak strain of VanA *E. faecium* being identified. However, outwith the outbreak situation, GRE isolates were more diverse in nature, with only small

clusters of related isolates being identified, and many deemed to be unrelated. PFGE analysis of the 94 isolates from the five-month study of enterococcal epidemiology revealed a heterogeneous enterococcal population, with only small clusters of related isolates recognized. Whilst the two VanA isolates arising from this study were unrelated to the nosocomial strains, some glycopeptide-sensitive enterococci were shown to be related to nosocomial GRE isolates.

Despite the ready transfer of glycopeptide resistance from the majority of the 48 nosocomial VanA GRE, attempts to identify plasmids harbouring Tn*1546* were unsuccessful. Despite this, the results of PFGE and hybridization studies suggested that many of the VanA *E. faecium* isolated from Edinburgh harbour a Tn*1546*-bearing plasmid of approximately 160-kilobases in size.

The Tn*1546*-related elements, responsible for conferring resistance in the 48 nosocomial VanA GRE, were assessed for diversity. Whilst complete characterization was not possible for all VanA elements, the majority could be assigned to one of ten different Tn*1546*-types on the basis of insertion and deletion events. Different Tn*1546*-types varied in their geographical distribution. The insertion sequence IS*1542* and an IS*1216V*-related element were identified within the *orf2-vanR* and *vanX-vanY* intergenic regions respectively. Further variation, at least partly attributable to the presence of an IS*1216V*-related element, was common within the *orf1/orf2* regions of the transposon. A hybrid promoter located upstream of *vanR* was identified, being generated by the insertion of IS*1542* immediately adjacent to the native -10 (TATAAT) box of the *vanR* promoter. This hybrid promoter is thought to be responsible for moderate-level constitutive expression of the glycopeptide resistance genes that was seen in those isolates harbouring the IS*1542* insertion within the VanA transposon. This hybrid promoter supplements, rather than replaces, the normal inducible expression from the native *vanR* promoter that follows glycopeptide challenge. A media-dependent increase in the level of teicoplanin resistance was seen in those strains harbouring the hybrid promoter. Whilst it is thought that this increase is attributable to the hybrid promoter, the exact mechanism is unclear.

In summary, this study reveals a heterogeneous GRE population within Scottish hospitals, both in terms of chromosome analysis by PFGE, and of genotyping of the

VanA resistance transposon. In so doing, a novel mechanism of *van* gene expression is described, which may, under certain circumstances, enhance the level of teicoplanin resistance.

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# Declaration

The experiments and composition of this thesis are the work of the author unless otherwise stated.

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# Publications and Presentations

* Brown, A.R., Amyes, S.G.B., Paton, R., Plant, W.D., Stevenson, G.M., Winney, R.J. & Miles, R.S. 1998. Epidemiology and control of vancomycin-resistant enterococci (VRE) in a renal unit. *Journal of Hospital Infection* **40**, 115-124.

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Brown, A.R., Townsley, A.C. & Amyes, S.G.B. Diversity of Tn*1546* elements in clinical isolates of glycopeptide-resistant enterococci from Scottish hospitals.
- Submitted to *Antimicrobial Agents and Chemotherapy* (July, 2000)

* Brown, A.R. & Amyes, S.G.B. 2000. Tn*1546* heterogeneity in VanA enterococci from Scotland: Correlation between genotype and teicoplanin resistance, abstr. 95. *In* Program and abstracts of the 1st International ASM Conference on Enterococci: Pathogenesis, Biology and Antibiotic Resistance. American Society for Microbiology, Washington, D.C.

* Copies of these publications are included after the appendices.
All are included with the permission of the publisher.

# Abbreviations

| | | |
|---|---|---|
| Ala | - | alanyl or alanine |
| ARI | - | Aberdeen Royal Infirmary |
| BGH | - | Borders General Hospital |
| BHI(A) | - | brain-heart infusion (agar) |
| $Bla^+$ | - | beta-lactamase-producing |
| bp | - | base pairs |
| CFU | - | colony-forming unit |
| DNA | - | deoxyribonucleic acid |
| dNTP | - | deoxynucleoside triphosphate |
| dTTP | - | 2'-deoxyribothymidine triphosphate |
| dUTP | - | 2'-deoxyribouridine triphosphate |
| ECH | - | Edinburgh City Hospital |
| ECL | - | enhanced chemiluminescence |
| EVREM | - | epidemic vancomycin-resistant *Enterococcus faecium* |
| GI | - | gastrointestinal |
| Glu | - | glutamate |
| GRE | - | glycopeptide-resistant enterococci |
| GSE | - | glycopeptide-sensitive enterococci |
| GWI | - | Western Infirmary, Glasgow |
| HLGR | - | high-level gentamicin resistance |
| HLSR | - | high-level streptomycin resistance |
| IS | - | insertion sequence |
| ISTA | - | isosensitest agar |
| IR | - | inverted repeat |
| kb | - | kilobases |
| L-PCR | - | long-template PCR |
| Lac | - | lactate |
| LTA | - | lipoteichoic acid |
| Lys | - | lysine |
| MHA | - | Mueller-Hinton agar |

| | | |
|---|---|---|
| MIC | - | minimum inhibitory concentration |
| min | - | minutes |
| MON | - | Monkland's Hospital, Airdrie |
| MRSA | - | methicillin-resistant *Staphylococcus aureus* |
| NIN | - | Ninewells Hospital, Dundee |
| P-VanR | - | phosphorylated VanR protein |
| PBP | - | penicillin-binding protein |
| PCR | - | polymerase chain reaction |
| PFGE | - | pulsed-field gel electrophoresis |
| RIE | - | Royal Infirmary of Edinburgh |
| RNA | - | ribonucleic acid |
| rRNA | - | ribosomal ribonucleic acid |
| s | - | seconds |
| Ser | - | serine |
| U | - | units of enzyme |
| UDP | - | uridine diphosphate |
| UTI | - | urinary tract infection |
| UV | - | ultraviolet |
| WGH | - | Western General Hospital, Edinburgh |

# Chapter 1: Introduction

## 1.1 The *Enterococcus*

As recently as the early 1980s, enterococci were perceived "with the exception of endocarditis and rare cases of meningitis ... [as] not ... a major cause of serious infection" (Kaye, 1982). However, within the last decade enterococci have become firmly established as major nosocomial pathogens, causing a wide variety of human infections. They are the fourth leading cause of hospital-acquired infection and the third leading cause of bacteraemia in the United States (Jett *et al.*, 1994). The rapid emergence of antibiotic resistance amongst enterococci has undoubtedly contributed to their emergence as a major nosocomial pathogen, rendering them amongst the most difficult of organisms to treat.

### 1.1.1 An historical perspective

In 1899, MacCallum and Hastings described a fatal case of endocarditis and identified the causative agent as a facultative Gram-positive coccus that they named *Micrococcus zymogenes* (MacCallum & Hastings, 1899). They noted the organism as being "very hardy and tenacious of life", withstanding desiccation, temperatures in excess of 60°C, and many antiseptics. This was the first description of the biological properties and pathogenicity of the organism that would ultimately be known as *Enterococcus faecalis*.

By the early 1930s, enterococci were classed as group D streptococci on the basis of the serological typing scheme established by Rebecca Lancefield (Lancefield, 1933), which exploited differences in cell wall polysaccharide antigens. In 1937, Sherman divided the group D streptococci into two physiologically different groups.

*Streptococcus bovis* and *S. equinus* were placed in the viridans division, whilst *S. faecalis* and *S. faecium* were placed in the enterococcus division (Sherman, 1937).

In 1970, Kalina proposed that both *S. faecalis* and *S. faecium* be transferred to the genus '*Enterococcus*' (Kalina, 1970). However, it was not until 1984, when DNA-DNA and DNA-RNA hybridization studies showed a more distant relationship between these two species and other non-enterococcal streptococci, that such a transfer took place (Schleifer & Kilpper-Bälz, 1984). The genus name was thus adopted for the bacteria previously named *S. faecalis* and *S. faecium* (Schleifer & Kilpper-Bälz, 1984). Subsequently, novel organisms and other streptococci have followed *S. faecalis* and *S. faecium* into the genus *Enterococcus*.

### 1.1.2 *Enterococcus* species

Since the genus was established in 1984 with the descriptions of *Enterococcus faecalis* and *E. faecium*, a further 17 species have been added to the *Enterococcus* genus on the basis of chemotaxonomic and phylogenetic studies (Devriese *et al.*, 1993). Many of the more recent additions do not fit the typical reactions of the genus that were described by Sherman (Sherman, 1937). Instead, these additions were based on evidence provided by 16S rRNA sequencing studies. Members of the genus are listed in table 1.1.

Since the descriptions of *E. seriolicida* and *E. solitarius*, phylogenetic studies indicate they are more closely related to *Lactococcus garviae* and *Tetragenococcus* species respectively (Collins *et al.*, 1990; Eldar *et al.* 1996; Williams *et al.*, 1991).

**Table 1.1: List of *Enterococcus* species described.**

| Present name *Enterococcus* | Description of the species | Previous name |
|---|---|---|
| *faecalis* | Schleifer & Kilpper-Bälz (1984) | *Streptococcus faecalis* |
| *faecium* | Schleifer & Kilpper-Bälz (1984) | *Streptococcus faecium* |
| *avium* | Collins *et al.* (1984) | *Streptococcus avium* |
| *casseliflavus* | Collins *et al.* (1984) | *Streptococcus casseliflavus* |
| *gallinarum* | Collins *et al.* (1984) | *Streptococcus gallinarum* |
| *durans* | Collins *et al.* (1984) | *Streptococcus durans* |
| *malodoratus* | Collins *et al.* (1984) | *Streptococcus faecalis* subsp. *malodoratus* |
| *hirae* | Farrow & Collins (1985) | ND |
| *mundtii* | Collins *et al.* (1986) | ND |
| *pseudoavium* | Collins *et al.* (1989) | ND |
| *raffinosus* | Collins *et al.* (1989) | ND |
| *solitarius* | Collins *et al.* (1989) | ND |
| *cecorum* | Williams *et al.* (1989) | *Streptococcus cecorum* |
| *columbae* | Devriese *et al.* (1990) | ND |
| *saccharolyticus* | Rodrigues & Collins (1990) | *Streptococcus saccharolyticus* |
| *sulfureus* | Martinez-Murcia & Collins (1991) | ND |
| *seriolicida* | Kusuda *et al.* (1991) | ND |
| *dispar* | Collins *et al.* (1991) | ND |
| *flavescens* | Pompei *et al.* (1992) | ND |

ND, New description

Adapted from Devriese *et al.* (1993)

In addition to identifying *Enterococcus* species, comparative 16S rRNA sequence analysis has also revealed the presence of several 'species groups' within the genus (Williams *et al.*, 1991). There are currently three species groups: the *E. faecium* group (*E. faecium, E. durans, E. hirae* and *E. mundtii*), the *E. avium* group (*E. avium, E. pseudoavium, E. raffinosus* and *E. malodoratus*), and the *E. casseliflavus/E. gallinarum* group. In general, members of a species group have >99% 16S rRNA sequence similarity with one another. It was proposed that *E. flavescens* would join the *E. casseliflavus/E. gallinarum* species group, although no 16S rRNA sequencing data of *E. flavescens* were available at the time (Devriese *et al.*, 1993). It has since been suggested, on the basis of 16S rDNA sequence analysis, that *E. flavescens* and *E. casseliflavus* comprise a single species (Patel *et al.*, 1998). The remaining enterococcal species, including the type species *E. faecalis*, form distinct lineages.

### 1.1.3 Identification of the *Enterococcus*

Whilst current tests, in general, enable identification of those *Enterococcus* species which most frequently occur in humans, many of the more recent enterococcal species cannot readily be identified by such tests. The 'original' *Enterococcus* species, *E. faecalis* and *E. faecium*, have several common characteristics that enable them to be distinguished from other catalase-negative, Gram-positive facultative cocci. These include the presence of Lancefield group D antigen, and their ability to grow at both 10 and 45°C, at pH 9.6, and in 6.5% sodium chloride broth (Schleifer & Kilpper-Bälz, 1984). However, many of the new enterococcal species fail to react with Lancefield group D antisera and fail to grow in the conditions that are characteristic of *E. faecalis* and *E. faecium* (Collins *et al.*

1989; Devriese *et al.*, 1990; Facklam & Collins, 1989; Martinez-Murcia & Collins, 1991). Furthermore, it is not only enterococci that may give positive reactions in these tests, with several streptococcal species, lactococci, pediococci, aerococci and leuconostocs liable to give positive reactions (Facklam *et al.*, 1989).

Other routinely applied tests include aesculin hydrolysis in conjunction with resistance to 40% bile, tests for urease, β-glucosidase and β-glucuronidase activities, and an array of carbohydrate acidification tests (Devriese *et al.*, 1993). Whilst none of these tests, or their combinations, are unique to enterococci, they remain valid tests to use when seeking only the classical *Enterococcus* species.

### 1.1.4 Natural habitat of enterococci

Enterococci are commensals of the gastrointestinal (GI) tract, being found in the intestine of nearly all animals, from cockroaches to humans. In human faeces they are typically found in numbers of $10^5$-$10^7$ CFU per gram (Jett *et al.*, 1994), being the predominant Gram-positive coccus in stools. They are, however, found in a number of environments, being readily recovered from a variety of outdoor sources (Hancock & Gilmore, 2000; Huycke *et al.*, 1998). This is likely to be attributable to their ability to persist in the environment following its contamination by sewage or animal excrement.

Whilst enterococci can also colonize the oral cavity and vaginal tract, their recovery from these sites is relatively rare (< 20% of cases) (Huycke *et al.*, 1998).

## 1.2 Enterococcal infections

*E. faecalis* accounts for approximately 80% of human enterococcal infections, with the majority of the remainder caused by *E. faecium* (Huycke *et al.*, 1998). This disparity between the two species may reflect the varying natural abundance of *E. faecalis* and *E. faecium*; *E. faecium* viable counts in human faeces are, on average, 100-fold lower than those of *E. faecalis* (Noble, 1978). An alternative explanation for the preponderance of *E. faecalis* infections is that this species may have enhanced virulence over *E. faecium*. Many virulence factors have only been reported in *E. faecalis* (see section 1.3), although it is likely that further investigation of *E. faecium* will reveal virulence determinants not yet identified in that species. Infections caused by enterococcal species other than *E. faecalis* and *E. faecium* are rare.

A significant risk factor for enterococcal infection is the use of broad-spectrum antibiotics such as third-generation cephalosporins and other antibiotics with significant activity against anaerobes, including metronidazole, imipenem and clindamycin (Edmond *et al.*, 1995; Graninger & Ragette, 1992; Livornese *et al.*, 1992). Such broad-spectrum therapy enables intestinal overgrowth of enterococci. Other risk factors for infection include prolonged hospitalization, a high severity of illness, intra-abdominal surgery and exposure to contaminated equipment or environmental surroundings (Edmond *et al.*, 1995; Graninger & Ragette, 1992; Livornese *et al.*, 1992; Wells *et al.*, 1990).

The most common enterococcal infections are urinary tract infections, bacteraemia, endocarditis and intra-abdominal infections.

### 1.2.1 Urinary tract infections (UTIs)

Over the last two decades, the proportion of UTIs caused by enterococci has risen significantly, in some cases being reported as the second most common cause of nosocomial UTI (Felmingham *et al.*, 1992; Morrison & Wenzel, 1986). It has recently been estimated that enterococci account for 110,000 UTIs annually in the United States (Huycke *et al.*, 1998). It is thought this increase in enterococcal UTIs is attributable to increasing use of urethral catheters and broad-spectrum antibiotic therapy, particularly the increasing use of cephalosporins (Felmingham *et al.*, 1992). The bladder, kidney and prostate are common infection sites, particularly in those patients with urinary tract abnormalities or indwelling catheters (Jett *et al.*, 1994). In one study, 95% of enterococcal UTI cases were preceded by urethral catheterization (Morrison & Wenzel, 1986).

### 1.2.2 Bacteraemia

Nosocomial surveillance data between October 1986 and April 1997 identified enterococci as the third most common cause of nosocomial bacteraemia, accounting for 12.8% of all isolates (Jarvis *et al.*, 1997). The ability of enterococci to translocate across intact intestinal epithelia is thought to lead to many bacteraemias with no identifiable source (Wells *et al.*, 1990). Many cases of enterococcal bacteraemia do, however, have an identifiable source, including intravenous lines, abscesses, UTIs and contaminated hospital equipment (Graninger & Ragette, 1992; Livornese *et al.*, 1992).

The primary risk for enterococcal bacteraemia is the use of broad-spectrum antibiotics which leads to intestinal overgrowth of enterococci, with bacteraemia

developing following growth to high numbers (Edmond *et al.*, 1995; Livornese *et al.*, 1992; Wells *et al.*, 1990).

### 1.2.3 Endocarditis

One of the most significant enterococcal infections, associated with considerable morbidity and mortality, is infective endocarditis. Enterococci are the third most common cause of infective endocarditis, behind streptococci and *Staphylococcus aureus* (Megran, 1992). When the source of infection is identifiable it is most frequently the genitourinary tract, usually attributable to either urinary tract infection or diagnostic/operative procedures (Kaye, 1985; Megran, 1992).

Due to the prevalence of degenerative valvular disease and genitourinary conditions in the elderly, enterococcal endocarditis tends to be a disease of older individuals, with a mean age of more than 60 years (Besnier *et al.*, 1994).

### 1.2.4 Intra-abdominal infections

Enterococci alone have little potential for causing intra-abdominal infections or soft tissue infections, with only a limited ability to infect in pure culture (Jett *et al.*, 1994). However, when enterococci are mixed with an otherwise avirulent anaerobic microorganism the infection is much more severe (Onderdonk *et al.*, 1976). Such microbial synergy between enterococci and anaerobes is well documented, although the mechanism by which it occurs is not understood (Brook, 1988; Hite *et al.*, 1949; Matlow *et al.*, 1989; Onderdonk *et al.*, 1976). Despite the accepted idea of microbial synergy, the exact role played by enterococci in such mixed infections, and their clinical significance, remains controversial. Whilst some advocate the administration

of anti-enterococcal therapy in the treatment of such mixed infections, others regard the enterococci as being non-pathogenic and consider anti-enterococcal therapy unnecessary (Brook, 1988).

### 1.2.5 Other enterococcal infections

Enterococci are frequently isolated from burn wound infections and infections of indwelling foreign devices such as intravascular catheters. Whilst they are also known to infect other sites, including the central nervous system, lungs, ears and eyes, such infections occur less frequently (Jett *et al.*, 1994).

## 1.3 Molecular basis of enterococcal disease

Many aspects of enterococcal pathogenicity are poorly understood. One of the key issues remaining unanswered is how, in a nosocomial setting, multiply-resistant enterococcal isolates that have been exogenously acquired appear to out-compete the indigenous enterococci that are already present. Many studies have attempted to address this issue, comparing the potential virulence factors expressed by infection-derived and non-infection-derived isolates.

Most attention has focussed on the expression of cell surface adhesion molecules and extracellular products that may have a role in virulence.

### 1.3.1 Cell surface adhesion molecules

Several studies have attempted to identify cell surface molecules that are enriched in infection-derived strains and thus may have a role in enterococcal adherence and subsequent infection. Lowe *et al.* described *E. faecalis* antigen A (EfaA), antibodies

against which were only seen in individuals with enterococcal endocarditis (Lowe *et al.*, 1995). Thus they hypothesized that EfaA may function as an important adhesin in endocarditis, the molecule showing significant homology to other streptococcal adhesins. There are, however, no experimental data to support this hypothesis.

A common finding of many studies, including that by Lowe and colleagues, is that the expression of many *E. faecalis* adhesion molecules is serum-dependent. In one study, whilst urinary isolates of *E. faecalis* adhered more readily to urinary tract epithelial cells than to heart cells, adherence of UTI isolates to cardiac cells was enhanced following growth in serum (Guzman *et al.*, 1989; Guzman *et al.*, 1991). Such observations led to the hypothesis that UTI isolates that establish a bacteraemic infection through invasion of the kidneys undergo alterations in their surface antigen expression following persistence in the blood (Guzman *et al.*, 1989). This may then result in enhanced adhesion to cardiac cells, enabling infection of the endocardium. Such a process could at least partly explain the long-established link between UTIs and endocarditis.

Aggregation substance is a plasmid-encoded cell-surface protein that is produced in response to sex pheromone secretion by recipient cells. Expression of aggregation substance causes aggregation of donor and recipient cells, thus facilitating plasmid transfer (Dunny *et al.*, 1995). Kreft *et al.* demonstrated that aggregation substance mediates binding, not only to other prokaryotic cells, but also to eukaryotic cells, by way of an amino acid motif that facilitates binding to integrins (Kreft *et al.*, 1992). They also demonstrated that expression of aggregation substance could be induced by unidentified serum components. Subsequent studies have supported this finding,

demonstrating aggregation substance-promoted adherence and internalization to eukaryotic cells (Sartingen *et al.*, 1998; Suessmuth *et al.*, 1998).

### 1.3.2 Extracellular products of enterococci

Enterococci secrete few known extracellular products, however two have been identified as being potential virulence factors in *E. faecalis*: cytolysin and gelatinase.

Haemolysin activity in *E. faecalis* was first reported in 1934 (Todd, 1934). On the basis of its broad target cell range (which includes eukaryotic and prokaryotic cells) the term haemolysin has now been superseded by the more general term, cytolysin (Jett *et al.*, 1994). Cytolysin lyses human, horse and rabbit erythrocytes, as well as having bacteriocin activity against many Gram-positive organisms (Basinger & Jackson, 1968). A higher incidence of cytolysin activity has been reported in clinical isolates of *E. faecalis* over faecal isolates (Ike *et al.*, 1987). The role of cytolysin in the virulence of enterococcal infection has also been well documented in animal models (Chow *et al.* 1993; Ike *et al.*, 1984; Jett *et al.*, 1992). However, many clinical strains are non-haemolytic, thus indicating that whilst cytolysin may play a role in virulence, it is certainly not an essential prerequisite.

Gelatinase, an extracellular metalloendoprotease, can hydrolyse gelatin, collagen, casein, haemoglobin and bioactive peptides (Makinen *et al.*, 1989). The medical importance of such metalloendoproteases is well-documented (Hase & Finkelstein, 1993), the enzymes frequently being associated with inflammatory processes and as potential virulence factors. Sequence analysis of the *E. faecalis* gelatinase gene, *gelE*, reveals significant homology to the elastase of *Pseudomonas aeruginosa*

(Fukushima *et al.*, 1989; Su *et al.*, 1991), and some evidence suggests gelatinase may contribute to the virulence of *E. faecalis* (Coque *et al.*, 1995; Dupont *et al.*, 1998).

### 1.3.3 Enterococci and the host immune system

#### 1.3.3.i Inflammatory response to enterococcal infection

Acute inflammation is a major pathological change associated with enterococcal infection. One of the factors involved in eliciting this inflammatory response is lipoteichoic acid (LTA), also known as group D streptococcal antigen (Toon *et al.*, 1972; Wicken *et al.*, 1963).

Bhakdi and colleagues demonstrated enterococcal LTA to be a potent inducer of monokine production, stimulating production to a higher level than that achieved by the LTA of other Gram-positives, and to a level similar to those achieved following exposure to Gram-negative LPS (Bhakdi *et al.*, 1991). Tsutsui *et al.* also demonstrated the stimulation of tumour necrosis factor (TNF) and interferon by enterococcal LTA (Tsutsui *et al.*, 1991).

The pheromones of *E. faecalis* are chromosomally encoded peptides (7-8 amino acids in length) that promote conjugative transfer of plasmid DNA. They have also been identified as pro-inflammatory mediators. Pheromone-responsive plasmids encode a peptide that acts as a competitive inhibitor of its corresponding pheromone. Some, but not all, pheromones and their peptide inhibitors are chemotactic for human and rat polymorphonuclear leucocytes *in vitro*, as well as stimulating granule enzyme secretion and respiratory burst (Ember & Hugli, 1989). Binding assays have shown these peptides to bind to the neutrophil receptors for formylmethionyl peptides, a

major class of bacterial chemotactic agents (Ember & Hugli, 1989; Sannomiya *et al.*, 1990).

### 1.3.3.ii Immune evasion

For pathogens to maintain an infection, they must successfully evade both specific and non-specific host defence mechanisms. Studies into the ability of enterococci to evade the host immune system suggest possible roles for some virulence traits, particularly in the protection against phagocytic cells. A particularly significant response to enterococcal infection is polymorphonuclear leucocyte-mediated killing that is primarily dependent on complement activation (Arduino *et al.*, 1994). Whilst strains capable of expressing gelatinase, cytolysin or aggregation substance appeared no more resistant to phagocytosis by neutrophils than strains lacking these traits (Arduino *et al.*, 1994; Jett *et al.*, 1994), studies have highlighted a potential protective role for aggregation substance against phagocytosis by macrophages (Suessmuth *et al.*, 1998). It should be noted, however, that the neutrophil-killing assay was performed under conditions that may not have supported the expression of cytolysin, gelatinase and aggregation substance, and so a potential protective role cannot be ruled out.

Peroxide and superoxide dismutase enzymes produced by enterococci may promote their survival within macrophages, catalysing hydrogen peroxide and superoxide that is found in phagolysosomes (Jett *et al.*, 1994).

Finally, a novel surface protein, Esp, which is enriched in infection-derived *E. faecalis* strains, may play a role in immune evasion. The structural gene allows for

alternate forms of Esp expression, generating variation at the bacterial cell surface that may aid immune evasion (Shankar *et al.*, 1999).

## 1.4 Antibiotic resistance in enterococci

The past two decades have witnessed the rapid emergence of multi-drug resistant enterococci, with the result that they are now firmly established as major nosocomial pathogens. Whilst *E. faecalis* causes the vast majority of human enterococcal infections, it is with *E. faecium* that the problem of multi-drug resistance is most prominent (Huycke *et al.*, 1998). Although *E. faecalis* exhibits many virulence traits, it is a species more likely to retain sensitivity to one or more effective antibiotics. However, *E. faecium*, whilst virtually devoid of known virulence traits, is more likely to be multi-drug resistant, with resistance now encompassing those antibiotics previously considered drugs of last resort (Huycke *et al.*, 1998).

Whilst enterococci are intrinsically resistant to many antibiotics, it is their propensity to acquire antibiotic resistance determinants through the exchange of plasmids and conjugative transposons which has rendered some clinical isolates resistant to all standard therapies.

### 1.4.1 The genetics of antibiotic resistance in enterococci

Enterococci can acquire antibiotic resistance determinants by three main methods: pheromone-responsive plasmids, broad host-range plasmids and conjugative transposons. Each of these play a significant role in the movement of resistance genes.

### 1.4.1.i Pheromone-responsive plasmids

The phermone-responsive plasmids of *E. faecalis* are perhaps the most extensively studied of the three mechanisms for gene transfer within enterococci (Clewell, 1981; Dunny *et al.*, 1995; Murray, 1998). *E. faecalis* secrete chromosomally-encoded pheromones (small peptides) which show specificity for different types of plasmids. When a potential donor cell containing a pheromone-responsive plasmid comes into contact with its corresponding pheromone, aggregation substance (encoded by the pheromone-responsive plasmid) is expressed on the surface of the cell. This facilitates aggregation (clumping) of donor and recipient cells, enabling highly efficient plasmid transfer. Transfer of pheromone-responsive plasmids can occur at frequencies as high as $5 \times 10^{-1}$, and occurs efficiently in both broth and filter matings. Once the recipient cell has acquired this particular plasmid, the synthesis of the corresponding pheromone is shut off to prevent self-clumping.

### 1.4.1.ii Broad host-range plasmids

Less well understood is the conjugation of broad host-range plasmids (Clewell, 1981; Murray, 1998). Unlike the pheromone-responsive plasmids that are restricted to *E. faecalis*, broad host-range plasmids occur across the *Enterococcus* genus, and have the potential to transfer to other Gram-positive organisms, including *Staphylococcus aureus*. This conjugation system is much less efficient than the pheromone-induced conjugation system, with filter matings resulting in much higher transfer frequencies than broth matings (Murray, 1998).

### 1.4.1.iii Conjugative transposons

The third method of conjugation is that of the conjugative transposons. Conjugative transposons are DNA elements that are normally integrated into the bacterial genome, but that have the capacity for intercellular transposition through a process of excision, conjugation and re-integration (Clewell & Gawronburke, 1986; Murray, 1998). They have a broad-host range and are found in Gram-positive and Gram-negative organisms.

One of the first conjugative transposons identified was Tn*916*, described in *E. faecalis* and carrying the tetracycline resistance determinant *tetM* (Franke & Clewell, 1981). Many other conjugative transposons have since been described, many of these being related to Tn*916* (Salyers *et al.*, 1995). The majority of conjugative transposons carry the tetracycline resistance determinant and as such have played a significant role in its dissemination.

### 1.4.2 Antibiotic resistance in enterococci

Most enterococci are intrinsically resistant to a wide variety of antibiotics, including cephalosporins, the anti-staphylococcal penicillins, and clinically achievable concentrations of aminoglycosides and clindamycin (Gray & Pedler, 1992; Murray, 1998). The activity of trimethoprim-sulphamethoxazole is, at best, controversial, with *in vivo* studies demonstrating poor efficacy of this antibiotic combination (Chenoweth *et al.*, 1990; Grayson *et al.*, 1990). This inherent *in vivo* resistance may be attributable to the ability of enterococci to utilize exogenous folates (Zervos & Schaberg, 1985), although the ability of *E. faecalis* to do so has been disputed (Hamilton-Miller & Purves, 1986).

In addition to these inherent resistance traits, enterococci have readily acquired plasmid or transposon-mediated resistance to various other antibiotics, including chloramphenicol, tetracyclines, macrolides, and high-level resistance to both clindamycin and trimethoprim (Murray, 1998). Target site alterations in GyrA and ParC have rendered many available fluoroquinolones unsuitable for treatment of enterococcal infections (El-Amin *et al.*, 1999; Kanematsu *et al.*, 1998; Tankovic *et al.*, 1996).

The scope of these intrinsic and acquired resistance determinants, which are summarized in table 1.2, has left few reliable therapeutic options for multi-drug resistant enterococci. In cases of serious enterococcal infection, the optimal therapeutic option is the synergistic combination of a cell wall-active agent (β-lactam or glycopeptide) with an aminoglycoside (Hewitt *et al.*, 1966; Moellering & Weinberg, 1971). The emergence of high level resistance to these antibiotics has, however, compromised this combination.

### 1.4.3 Penicillin resistance in enterococci

In comparison with streptococci, enterococci are relatively resistant to the penicillins, a feature attributable to low affinity of the penicillin-binding proteins (PBPs) (Gray & Pedler, 1992). *E. faecalis* MICs typically range from 1 – 8 mg/L, with *E. faecium* displaying even higher levels of resistance owing to the PBPs of *E. faecium* having significantly lower affinities for the antibiotics than the PBPs of *E. faecalis* (Huycke *et al.*, 1998; Murray, 1998).

**Table 1.2: Summary of antibiotic resistance in enterococci**

| ANTIBIOTIC | MECHANISMS OF RESISTANCE | REFERENCES |
|---|---|---|
| **β-lactam antibiotics** | Alteration to penicillin-binding protein profile | Fontana *et al.* (1996); Williamson *et al.* (1985) |
| | β-lactamase production | Murray & Mederski-Samoraj (1983); Murray (1992) |
| **Aminoglycosides** | Impermeability | Moellering & Weinberg (1971) |
| | Aminoglycoside-modifying enzymes | Horodniceanu *et al.* (1979); Ferretti *et al.* (1986); Chow *et al.* (1997a) |
| **Fluoroquinolones** | GyrA and ParC target site alterations | Tankovic *et al.* (1996); El-Amin *et al.* (1999); Kanematsu *et al.* (1998) |
| **Glycopeptides** | Target site alteration through cell wall modification | Woodford (1998) |
| **Trimethoprim** | Use of exogenous folates *in vivo* | Zervos & Schaberg (1985); Grayson *et al.* (1990) |
| | Acquisition of dihydrofolate reductase genes | Coque *et al.* (1999) |
| **Tetracyclines** | Ribosomal protection attributable to Tet determinants | Bentorcha *et al.* (1991); Bentorcha *et al.* (1992); Charpentier *et al.* (1994) |
| **Chloramphenicol** | Chloramphenicol acetyl transferases | Triuecuot *et al.* (1993) |
| **MLS antibiotics** (Macrolide-Lincosamide-Streptogramin) | $MLS_b$ cross-resistance by methylation of 23S rRNA (*ermB*) | Portillo *et al.* (2000); Schmitz *et al.* (2000) |
| | Inactivation of lincosamide antibiotics (*linB*) | Bozdogan *et al.* (1999) |
| | Efflux of macrolides and streptogramin B (*msrC*) | Portillo *et al.* (2000) |
| | Inactivation of stretogramin group A (*satA*, *satG*) | Rende-Fournier *et al.* (1993); Werner & Witte (1999) |

Furthermore, enterococci are tolerant to the bactericidal effect of cell wall-active agents, both β-lactam and non-β-lactam antibiotics (Fontana *et al.*, 1990; Krogstad & Parquette, 1980). This tolerance to killing is most likely attributable to a defective autolytic enzyme system (Fontana *et al.*, 1990), although it has been suggested that rather than tolerance being due to an inherent defect, it may be acquired following exposure to the antibiotics (Hodges *et al.*, 1992). This tolerance to killing is the reason why systemic enterococcal infections require the synergistic combination of a cell wall-active agent and an aminoglycoside.

As with any synergistic combination of antibiotics, synergy is abolished by the emergence of high-level resistance to either of the antibiotics in the combination. High-level penicillin resistance in enterococci can arise through two distinct mechanisms: β-lactamase production and PBP overproduction.

### 1.4.3.i β-lactamase-producing enterococci

β-lactamases in enterococci are primarily, though not exclusively, restricted to *E. faecalis* (Murray, 1992). The first β-lactamase-producing ($Bla^+$) strain of *E. faecalis* was reported in 1983 (Murray & Mederski-Samoraj, 1983). The enterococcal penicillinase has subsequently been shown to be identical to the staphylococcal type A penicillinase, and has greatest activity against penicillin, ampicillin and the ureidopenicillins (Murray, 1992). In sensitivity tests, the levels of resistance conferred by enterococcal β-lactamases are very dependent on the inoculum size, so much so that at routinely used inocula ($10^5$ CFU/mL), $Bla^+$ enterococci are often no more resistant to penicillin than other enterococci (Murray, 1992). Increasing the inoculum to $10^7$ CFU/mL can elevate penicillin MICs to 1000 mg/L or more

(Murray, 1992). Whilst an inoculum effect is characteristic of $Bla^+$ organisms, it is particularly exaggerated in enterococci, as other $Bla^+$ organisms are usually readily detectable at routinely used inocula.

Unlike staphylococci, the β-lactamase of enterococci does not appear to be released into the extracellular medium (Murray *et al.*, 1986). Instead, the enzyme remains cell-associated, most likely due to either a failure to be cleaved from its signal peptide sequence, or binding of the enzyme to other cellular components (Murray, 1992). The cell-associated nature of the enzyme somewhat contradicts the extreme inoculum effect that is characteristic of the enterococcal penicillinase. Usually this effect is attributable to the extracellular release of the enzyme. However, in the case of the enterococcal enzyme, it may rather be due to low-level expression of the enzyme (Zscheck & Murray, 1991).

Several sporadic outbreaks of β-lactamase-producing *E. faecalis* have been reported although these are largely restricted to the US (Murray *et al.*, 1991; Wells *et al.*, 1992). β-lactamase-producing enterococci remain uncommon in Europe, with typically less than 1% of *E. faecalis* isolates displaying ampicillin resistance (Iwen *et al.*, 1997; Klare *et al.*, 1999a).

### 1.4.3.ii Overproduction of penicillin-binding proteins (PBPs)

High-level penicillin resistance (≥200 mg/L) has also been reported in enterococci (primarily *E. faecium*) that do not produce β-lactamase enzymes (Bush *et al.*, 1989; Sapico *et al.*, 1989). This form of resistance has been associated with overproduction of a low affinity PBP (PBP5) that is seemingly able to perform the functions of the other PBPs (Fontana *et al.*, 1996; Williamson *et al.*, 1985). As well

as overproduction of PBP5, amino acid substitutions within the protein appear to cause a further decrease in its affinity for penicillin (Fontana *et al.*, 1996). Overproduction may be associated with a deletion in a region upstream of the *pbp5* gene that ordinarily negatively controls synthesis of PBP5 (Fontana *et al.*, 1996).

This mechanism of high-level penicillin resistance appears to be more common than resistance through β-lactamase production; typically over 70% of *E. faecium* isolates are ampicillin resistant (Iwen *et al.*, 1997; Klare *et al.*, 1999a).

### 1.4.4 Aminoglycoside resistance in enterococci

Enterococci are ordinarily relatively resistant to aminoglycosides due to impermeability, with gentamicin MIC values typically ranging from 4 to 64 mg/L (Gray & Pedler, 1992). The combination of a cell wall-active agent with an aminoglycoside, however, provides a synergistic bactericidal effect that is most likely attributable to increased uptake of the aminoglycoside in the presence of the cell wall-active agent (Moellering & Weinberg, 1971). In addition to resistance mediated by impermeability, *E. faecium* is intrinsically aminoglycoside resistant due to the activity of a chromosomal acetyltransferase enzyme that modifies the antibiotic (Chen, 1986).

Whilst high-level streptomycin resistance is sometimes mediated by alteration in ribosomal protein S12 (Eliopoulos *et al.*, 1984), high-level aminoglycoside resistance is usually due to plasmid-encoded aminoglycoside-modifying enzymes. Plasmid-encoded high-level streptomycin resistance (MIC >2000mg/L) was first identified in *E. faecalis* in 1970 (Moellering *et al.*, 1971). Such resistance, conferred by the production of streptomycin adenyltransferase, is now common in both *E. faecalis* and

*E. faecium* (Gray & Pedler, 1992). Nine years after the emergence of high-level streptomycin resistance, high-level gentamicin resistance (HLGR) was reported in *E. faecalis* (Horodniceanu *et al.*, 1979), associated with the production of a bifunctional 6'-aminoglycoside acetyltransferase 2"- aminoglycoside phosphotransferase enzyme that confers resistance to all clinically-available aminoglycosides, with the exception of streptomycin (Ferretti *et al.*, 1986). However, many HLGR strains also produce streptomycin adenyltransferase (Gray & Pedler, 1992).

Other aminoglycoside modifying enzymes include a 4',4"-aminoglycoside nucleotidyltransferase that confers resistance to tobramycin, kanamycin, neomycin and dibekacin (Carlier & Courvalin, 1990), and the gentamicin-modifying enzymes, encoded by the *aph(2")-1b*, *aph(2")-1c*, and *aph(2")-1d* genes, which confer clinically-significant gentamicin resistance (Chow *et al.*, 1997a; Chow, 2000).

High-level aminoglycoside resistance is now common in *E. faecalis* and *E. faecium*, with prevalence typically ranging from 20–50% (Iwen *et al.*, 1997; Klare *et al.*, 1999a; Watanakunakorn, 1989).

By the late 1980s, high-level penicillin resistance and high-level aminoglycoside resistance were increasing in prevalence amongst clinical enterococcal isolates. The previously reliable synergistic combination of a cell wall-active agent and an aminoglycoside was becoming increasingly compromised and therapeutic failures ensued (Fernandez-Guerrero *et al.*, 1988). The biggest surprise, however, came with the emergence of acquired vancomycin resistance, first reported in 1988 (Leclercq *et al.*, 1988; Uttley *et al.*, 1988). After 30 years of clinical use, many had been lulled into thinking that acquired resistance to vancomycin would not appear. However, its emergence and rapid dissemination amongst primarily nosocomial enterococci has

rendered some enterococcal infections untreatable by commercially available, clinically proven therapies, and has brought the prospect of a "post-antimicrobial era" (Cohen, 1992) somewhat closer.

## 1.5 The glycopeptide antibiotics

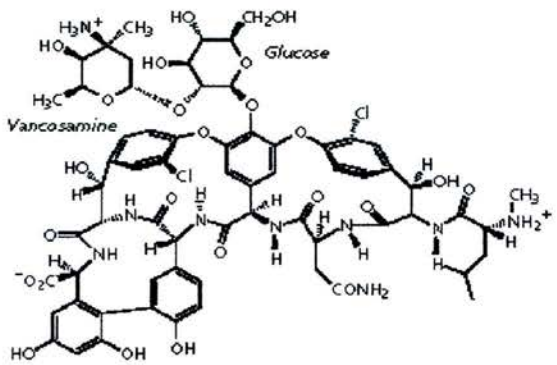
There are two clinically significant glycopeptide antibiotics in use today, vancomycin and teicoplanin. Vancomycin has been in use since the late 1950s, with its usage increasing rapidly throughout the 1980s and early 1990s as it became recognized as sometimes the only reliable antibiotic for the treatment of multiply resistant staphylococci (Kirst *et al.*, 1998; Wegener, 1998). However, vancomycin usage is associated with well-known side effects, including ototoxicity, nephrotoxicity, neutropenia and severe allergic reactions (van Laethem *et al.*, 1988). These disadvantages drove the investigation of other potential glycopeptide agents giving rise to teicoplanin, which is licensed for use in Europe. Both vancomycin and teicoplanin are produced naturally by the soil-living bacteria *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus* respectively (Marshall *et al.*, 1997; Parenti *et al.*, 1978).

A third glycopeptide, which will be discussed later, is avoparcin. Avoparcin was used extensively throughout Europe for over 20 years as a growth-promoting agent in animal livestock production. Its use within the European Union was withdrawn in 1997 owing to concerns over a potential risk to human health.

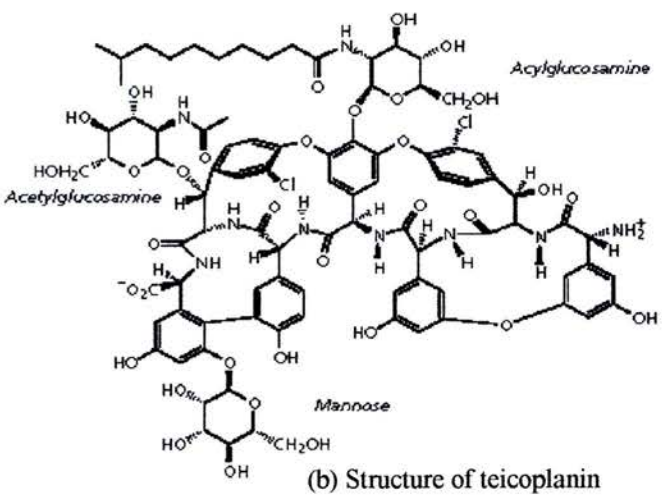
### 1.5.1 Activity spectrum of the glycopeptides

The glycopeptide antibiotics are large polar molecules and as such cannot penetrate the outer membrane of Gram-negative organisms (Reynolds, 1985). Their activity is thus restricted to Gram-positive organisms, both anaerobes and aerobes. The structures of vancomycin and teicoplanin are shown in figure 1.1.

**Figure 1.1: Structures of the glycopeptide antibiotics, vancomycin and teicoplanin** (From Gilpin & Milner, 1997)




(a) Structure of vancomycin




(b) Structure of teicoplanin

The spectrum of activity of vancomycin and teicoplanin, although similar, is not identical (Greenwood, 1988). Teicoplanin is generally more active against streptococci and Gram-positive anaerobes than is vancomycin, whilst vancomycin has the greatest activity against coagulase-negative staphylococci. The activity of the two agents is comparable against *S. aureus*, including those strains that are methicillin-resistant. The main indications for glycopeptide usage are in the treatment of MRSA infections, *Clostridium difficile* infections, and serious Gram-positive infections in patients allergic to β-lactams.

### 1.5.2 Mode of action of the glycopeptides

All glycopeptide antibiotics inhibit the latter stages of cell wall synthesis by forming complexes with peptidoglycan precursors. Within the cytoplasm of the cell, a D-alanyl-D-alanine (D-Ala-D-Ala) ligase produces the dipeptide D-Ala-D-Ala that is added to UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys (Neuhaus, 1962; Walsh, 1989). Once the peptidoglycan precursor is complete, it is translocated across the cytoplasmic membrane by a lipid carrier, and it is at this stage that the glycopeptides inhibit cell wall synthesis (Arthur *et al.*, 1996b). Following translocation of the precursors to the outer surface of the cytoplasmic membrane, the glycopeptides bind to the carboxy-terminal D-alanine residues of the cell wall precursors. This binding blocks the incorporation of the peptidoglycan precursors into the nascent cell wall by transglycosylation, and leads to the accumulation of cytoplasmic precursors. Binding of the antibiotics to D-Ala-D-Ala-terminating peptide stems within nascent peptidoglycan is also believed to inhibit cell wall synthesis through inhibition of the transpeptidase and carboxypeptidase steps of cell wall synthesis.

## 1.6 Glycopeptide resistance in enterococci

High-level resistance to the glycopeptides in clinical enterococcal isolates was first reported in 1988 by Uttley and colleagues (Uttley *et al.*, 1988). Further enterococcal isolates displaying a similar resistance phenotype were described later that same year (Leclercq *et al.*, 1988). These two publications described what was to become known as VanA phenotype glycopeptide resistance, characterized by high-level inducible resistance to both vancomycin and teicoplanin. Whilst predominantly found in *E. faecium*, VanA-type resistance also occurs in *E. faecalis* and occasionally in other enterococcal species (Woodford, 1998).

The year after the description of VanA-type glycopeptide resistance, a second phenotype was reported (Williamson *et al.*, 1989). This VanB phenotype is characterized by low to moderate levels of vancomycin resistance but susceptibility to teicoplanin, and is found predominantly in *E. faecalis* and *E. faecium* (Woodford, 1998).

VanA and VanB phenotype glycopeptide resistance are the two most clinically significant forms of glycopeptide resistance in enterococci. However, since their description, other glycopeptide resistance phenotypes have been described. VanC phenotype glycopeptide resistance is an intrinsic property of *E. casseliflavus*, *E. gallinarum* and *E. flavescens*, and is characterized by low-level resistance to vancomycin and susceptibility to teicoplanin (Dutka-Malen *et al.*, 1992; Navarro & Courvalin, 1994). The recently described VanD and VanE phenotypes are acquired resistance traits seen in *E. faecium* and *E. faecalis* respectively (Fines *et al.*, 1999; Perichon *et al.*, 1997). These five resistance phenotypes are summarized in table 1.3.

**Table 1.3: Characteristics of the glycopeptide resistance phenotypes**

| Characteristics | Glycopeptide Resistance Phenotype | | | | |
|---|---|---|---|---|---|
| | **VanA** | **VanB** | **VanC** | **VanD** | **VanE** |
| **Type of resistance** | Acquired | Acquired | Intrinsic | Acquired | Acquired |
| **Vancomycin MIC** | 64 → >1000 | 8 → >1000 | 8 → 32 | 64 → 128 | 16 |
| **Teicoplanin MIC** | 16 → > 1000 | 0.5 → 1 | 0.5 → 1 | 4 | 0.5 |
| **Expression** | Inducible | Inducible | Constitutive | Constitutive | Inducible |
| **Location of genes** | Plasmid or chromosomal | Chromosomal or plasmid | Chromosomal | Presumably chromosomal | Presumably chromosomal |
| **Transposon** | Tn*1546* | Tn*1547*, Tn*5382* | - | - | - |
| **Transferability** | Yes | Yes | No | No | No |
| **Terminal residues of precursors** | D-Ala-D-Lac | D-Ala-D-Lac | D-Ala-D-Ser | D-Ala-D-Lac | D-Ala-D-Ser |
| **Host species** | *Enterococcus* spp. | *Enterococcus* spp.<br>*Streptococcus bovis* | *E. casseliflavus*<br>*E. gallinarum*<br>*E. flavescens* | *E. faecium* | *E. faecalis* |
| **References** | Arthur *et al.* (1993) | Quintiliani Jr. & Courvalin (1996)<br>Carias *et al.* (1998) | Navarro & Courvalin (1994)<br>Dutka-Malen *et al.* (1992) | Perichon *et al.* (1997)<br>Casadewall & Courvalin (1999) | Fines *et al.* (1999)<br>Abadio Patino *et al.* (2000) |

A further two glycopeptide resistance gene clusters have been identified, termed *vanF* and *vanG*. The *vanG* resistance locus was identified in *E. faecalis* isolates that displayed the VanB phenotype but were negative by PCR for all the previously recognized *van* genes (McKessar *et al.*, 2000). No data is available concerning the nature of the terminal amino acid of the peptidoglycan precursors of these isolates. The genetic organization of the *vanG* locus is shown in figure 1.4 (page 39)

The *vanF* resistance locus is a cluster of five genes identified in *Paenibacillus popilliae*, a vancomycin-resistant organism that has been used as a biopesticide in the United States for over 50 years (Patel *et al.*, 2000). It has been proposed that the resistance genes in *P. popilliae* may have been precursors to those seen in GRE (see section 1.7.6).

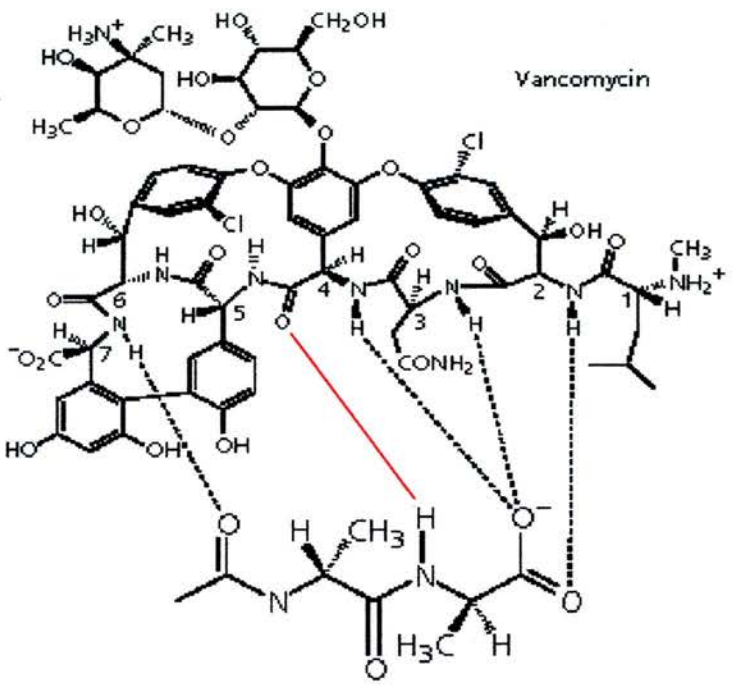
### 1.6.1 Molecular basis of glycopeptide resistance

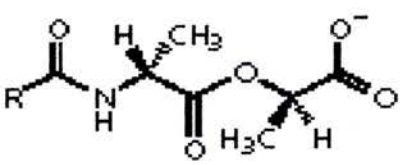
The five resistance phenotypes summarized in table 1.3 share the same basic mechanism of resistance. As discussed in section 1.5.2, the glycopeptides bind to the carboxy-terminal D-Ala residues of cell wall precursors, thus preventing their incorporation into the nascent peptidoglycan. Substituting the terminal D-Ala residue with either D-lactate (VanA, VanB and VanD phenotypes) or D-serine (VanC and VanE phenotypes) confers resistance (Arthur *et al.*, 1996b).

The D-lactate (D-Lac) substitution causes the replacement of a peptide bond by an ester bond within the cell wall precursor. This replacement causes the loss of a single hydrogen bond, that ordinarily forms between vancomycin and the cell wall precursor (Bugg *et al.*, 1991a). The loss of this hydrogen bond results in a 1000-fold decrease in affinity for vancomycin binding, thus resulting in vancomycin resistance

(Bugg *et al.*, 1991a). The binding of vancomycin to the terminal D-Ala-D-Ala residues via hydrogen bonds is illustrated in figure 1.2, alongside the structure of the D-Ala-D-Lac depsipeptide.

**Figure 1.2: Binding of vancomycin to D-Ala-D-Ala, and the structure of the altered precursor, D-Ala-D-Lac**



*C-terminus of the D-Ala-D-Lactate peptide*

Hydrogen bonds formed between vancomycin and the terminal D-Ala-D-Ala residues are illustrated, with the hydrogen bond that is lost by the substitution of D-Ala with D-lactate depicted in red. (Adapted from Gilpin & Milner, 1997)

In comparison with other antibiotic resistance traits that are conferred by a single gene product or mutation in a single gene, acquired glycopeptide resistance occurs by a complex mechanism involving a series of enzymatic reactions. VanA-type resistance, for example, discussed in depth in section 1.7, is conferred by seven different genes, involved in the regulation of resistance gene expression, the production of altered cell wall precursors (terminating in D-lactate) and the degradation of native cell wall and cell wall precursors (terminating in D-Ala) (Arthur *et al.*, 1996b). In general, a cell co-producing D-Ala-D-Ala and D-Ala-D-Lac-terminating cell wall precursors will not be glycopeptide resistant, as antibiotic will bind to those D-Ala-D-Ala-terminating precursors as they are translocated across the cytoplasmic membrane, thus sequestering the lipid carrier necessary for their translocation (Arthur *et al.*, 1996b).

### 1.6.2 Glycopeptide-dependent enterococci

In 1994, variants of glycopeptide-resistant *E. faecalis* and *E. faecium*, derived from both VanA and VanB phenotypes, were isolated from patients who had undergone vancomycin therapy (Fraimow *et al.*, 1994; Woodford *et al.*, 1994). These variants, known as glycopeptide-dependent enterococci, grow only in the presence of glycopeptide antibiotics or if supplied with the dipeptide D-Ala-D-Ala. This suggested that these variants are unable to produce the *ddl*-encoded ligase normally responsible for production of the D-Ala-D-Ala dipeptide. Recent studies have confirmed this, showing mutations in the *ddl* gene leading to either amino acid substitutions or deletions (van Bambeke *et al.*, 1999). With the impaired host D-Ala-D-Ala ligase, cell wall synthesis can only proceed in the presence of glycopeptides

that induce the VanA or VanB ligase, resulting in synthesis of the D-Ala-D-Lac depsipeptide.

Vancomycin-independent revertants can arise from previously dependent strains through two distinct mechanisms. Compensatory mutations in the *ddl* gene can restore ligase activity, or alternatively, a mutation in the glycopeptide-resistance regulatory genes can result in constitutive expression of the VanA/VanB ligase (van Bambeke *et al.*, 1999).

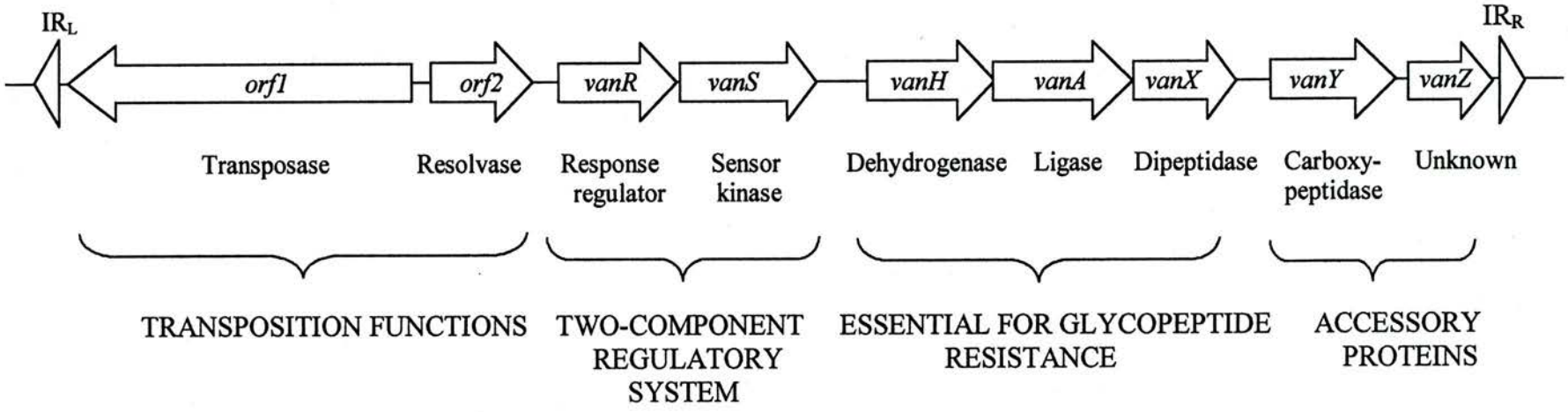
Whilst glycopeptide-dependent enterococci have been described as 'superbugs' (Farrag *et al.*, 1996), it is generally accepted that they are more of a curiosity than a major clinical concern, carrying no more implication than normal GRE, with the exception that they are not readily detected by standard screening techniques.

### 1.7 Tn*1546* – the VanA transposon

The genetic element responsible for conferring high-level glycopeptide resistance in *E. faecium* BM4147, one of the first clinical GRE isolates, was fully characterized in 1993 (Arthur *et al.*, 1993). The element, designated Tn*1546*, is now known to confer VanA phenotype glycopeptide resistance, with all VanA enterococci harbouring Tn*1546*-related elements.

Tn*1546* (10,851-bp) is a Tn*3*-related transposon encoding nine polypeptides involved in the regulation of resistance, the resistance mechanism itself, and the transposition functions of Tn*1546*. The basic structure of Tn*1546* is well conserved and is depicted in figure 1.3. Variation within Tn*1546*-related elements and the use of such variation as an epidemiological tool is discussed in section 1.9.

**Figure 1.3: Genetic map of Tn*1546***



### 1.7.1 ORF1 and ORF2: Transposition functions

Analysis of Tn*1546* and its transposition functions showed that it conformed to the Tn*3* family of transposons (Arthur *et al.*, 1993). Tn*3*-related transposons typically harbour two genes that are essential for transposition (encoding transposase and resolvase enzymes), and a *res* site that serves to complete the transposition process. The transposase enzyme mediates formation of a cointegrate intermediate during which the transposon is replicated. The resolvase enzyme then mediates site-specific recombination between the *res* sites of the two transposons thus resolving the cointegrate.

The *orf1* and *orf2* genes of Tn*1546* encode proteins that are structurally related to transposases and resolvases respectively (Arthur *et al.*, 1993). As in Tn*3*, the transposase and resolvase genes of Tn*1546* are transcribed in opposite directions and are separated by a putative *res* site (Arthur *et al.*, 1993). However, despite the structural similarities between Tn*1546* and Tn*3*, sequence similarity between the transposase and resolvase genes of the two transposons is low (around 30%) (Arthur *et al.*, 1993). Whilst Tn*1546* transposition has been shown to be replicative via cointegrate formation, studies in *E. coli* deficient in general recombination have failed to demonstrate ORF2-mediated resolution of cointegrates, suggesting the Tn*1546* resolvase is nonfunctional (Arthur *et al.*, 1993). Sequence alignment of ORF2 with other resolvase enzymes has revealed two apparent amino acid substitutions within ORF2 that may be responsible for its impaired resolvase activity.

### 1.7.2 VanR and VanS: Two-component regulatory system

VanR is related to members of the OmpR class of response regulators, whilst VanS is related to membrane-associated protein histidine kinases. Together they form a two-component regulatory system that activates expression of the *van* gene cluster in response to glycopeptide challenge (Wright *et al.*, 1993).

Gel shift experiments and DNaseI footprinting using phosphorylated VanR (P-VanR) have demonstrated P-VanR binding sites at both the *vanR* and *vanH* promoter regions, located upstream of *vanR* and within the *vanS-vanH* intergenic region respectively (Holman *et al.*, 1994). Whilst unphosphorylated VanR is capable of binding at these sites, phosphorylation to yield P-VanR greatly increases the binding affinity (Holman *et al.*, 1994). P-VanR has a markedly higher affinity for the *vanH* promoter region than the *vanR* promoter region, and has a protected footprint at the *vanH* promoter twice the size of the footprint at the *vanR* promoter. These findings are consistent with the oligomerization of P-VanR on the *vanH* promoter (Holman *et al.*, 1994).

Analysis of the P-VanR footprinted regions have led to the identification of a 12-bp consensus sequence that may act as a recognition site for P-VanR binding (Holman *et al.*, 1994). One copy of this consensus sequence is present within the *vanR* promoter region, whereas two copies are present at the *vanH* promoter. This further supports the oligomerization of P-VanR on the *vanH* promoter.

Whilst it was initially proposed that P-VanR binding had a negative effect on the *vanR* promoter and a positive effect on the *vanH* promoter (Holman *et al.*, 1994), subsequent studies have shown P-VanR binding to have a positive role at both promoters (Arthur *et al.*, 1997; Haldimann *et al.*, 1997). Thus P-VanR binding at the

*vanR* promoter establishes a positive amplification loop for *vanRS* expression, whilst binding at the *vanH* promoter activates cotranscription of the *van* gene cluster and thus expression of glycopeptide resistance.

### 1.7.3 VanH, VanA & VanX: Essential gene products

The remaining genes of Tn*1546*, *vanHAXYZ*, can be assigned to two functional groups: those which are essential for glycopeptide resistance (*vanH*, *vanA* and *vanX*) and those that encode non-essential accessory proteins (*vanY* and *vanZ*) (Evers *et al.*, 1996). Of the three essential gene products, two (VanH and VanA) are required for the production of the altered peptidoglycan precursors that terminate in D-Ala-D-Lac, whilst VanX is required for the elimination of D-Ala-D-Ala dipeptides, preventing their incorporation into peptidoglycan precursors.

The VanA protein shows significant amino acid sequence homology to the D-Ala-D-Ala ligases of *E. coli* (Dutka-Malen *et al.*, 1990b). Whilst VanA displays D-Ala-D-Ala ligase activity, the catalytic efficiency of the enzyme is considerably lower (ca. 100-fold) than those of the *E. coli* ligases (Bugg *et al.*, 1991b). Unlike other D-Ala-D-Ala ligases, VanA displays broad substrate specificity, capable of producing mixed dipeptides including D-alanine-D-methionine, D-alanine-D-phenylalanine and D-alanine-D-aminobutyrate (Bugg *et al.*, 1991b). These results led to the conclusion that the synthesis of a D-Ala-D-X compound by VanA enabled cell wall synthesis to proceed in the presence of glycopeptides. The exact nature of this X compound was established as a result of studies on the VanH protein and analysis of peptidoglycan precursors of glycopeptide-resistant and susceptible enterococci (Arthur *et al.*, 1991; Arthur *et al.*, 1992a; Bugg *et al.*, 1991a; Handwerger *et al.*, 1992). It is now known

that the preferred reaction catalysed by the VanA ligase enzyme is ester bond formation between D-alanine and D-lactate, forming the depsipeptide D-Ala-D-Lac (Arthur *et al.*, 1992a). This depsipeptide replaces D-Ala-D-Ala in the formation of peptidoglycan precursors. The VanH enzyme, a dehydrogenase that reduces 2-keto acids to D-hydroxy acids, provides the substrate for VanA by reducing pyruvate to D-lactate (Bugg *et al.*, 1991a).

As discussed in section 1.6.1, a cell co-producing D-Ala-D-Ala- and D-Ala-D-Lac-terminating peptidoglycan precursors will not be glycopeptide resistant. There must, therefore, be a mechanism for the degradation of cell wall precursors terminating in D-Ala-D-Ala. VanX, a D,D-dipeptidase, plays a key role in the elimination of such precursors. VanX hydrolyses the D-Ala-D-Ala dipeptide prior to its incorporation into the disaccharide pentapeptide precursors (Reynolds *et al.*, 1994). As well as serving to prevent the formation of D-Ala-D-Ala-terminating peptidoglycan precursors, the function of VanX recycles D-Ala, thus ensuring substrate for the VanA ligase.

### 1.7.4 VanY & VanZ: Accessory proteins

Downstream of the *vanHAX* gene cluster are the *vanY* and *vanZ* genes encoding non-essential accessory proteins.

The *vanY* gene encodes a D,D-carboxypeptidase that can remove the terminal D-alanine of peptidoglycan precursors terminating in D-Ala-D-Ala, thus generating the tetrapeptide precursor UDP-*N*-acetylmuramyl-L-Ala-γ-D-Glu-L-Lys-D-Ala (Arthur *et al.*, 1992b; Arthur *et al.*, 1994). Whilst VanY also displays carboxyesterase activity, hydrolysing the D-Ala-D-Lac-terminating precursors generated by VanH and VanA,

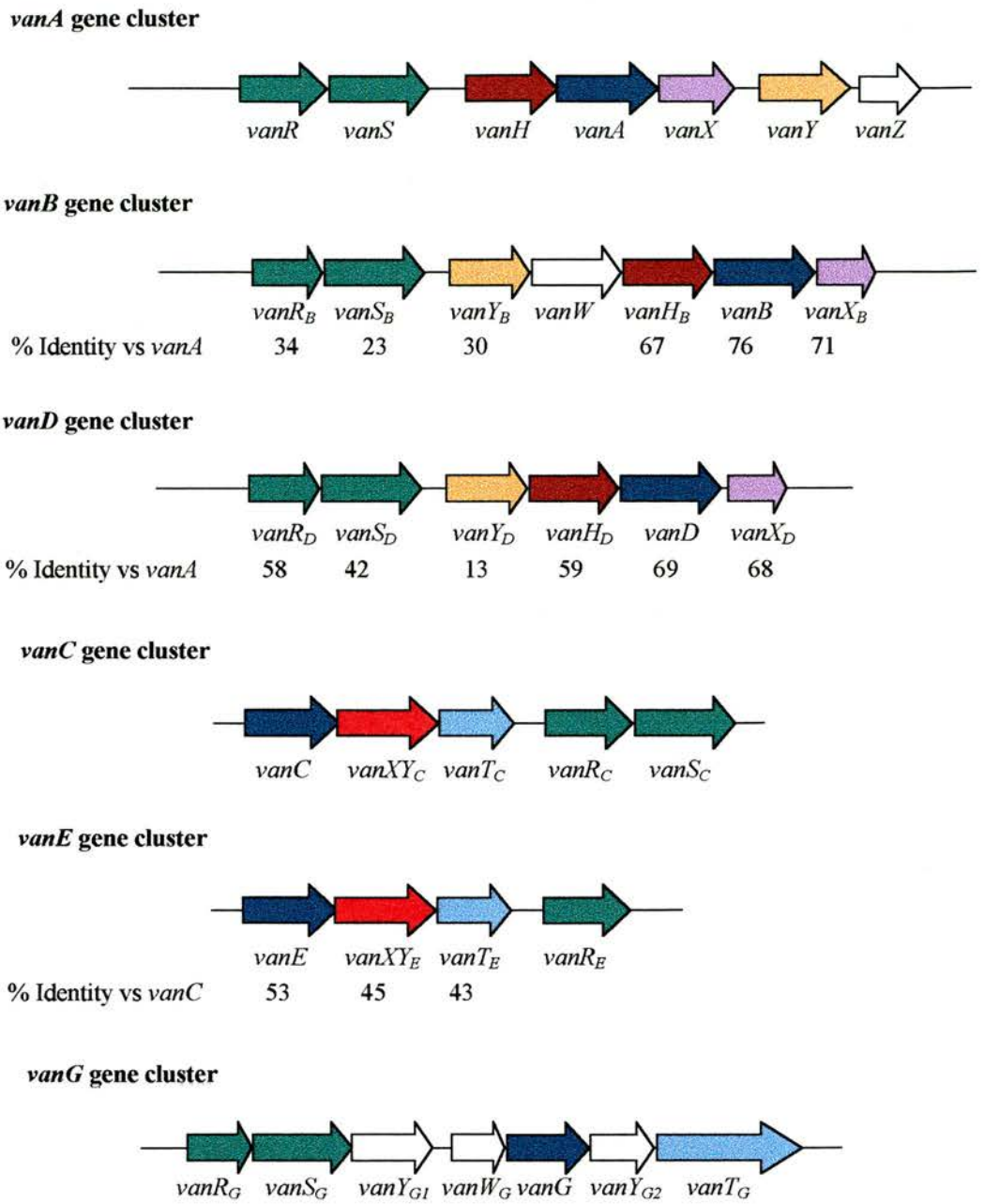
it is likely that its main role is as a 'back-up' mechanism, preventing the incorporation of D-Ala-D-Ala-terminating precursors into nascent peptidoglycan (Arthur *et al.*, 1994; Wright *et al.*, 1992). Whilst the VanX dipeptidase would ordinarily be expected to prevent the incorporation of D-Ala-D-Ala-terminating precursors by hydrolysis of the D-Ala-D-Ala dipeptide, it is feasible that in the presence of excess D-alanine some dipeptides are not hydrolysed by VanX. Following the incorporation of these residual D-Ala-D-Ala peptides into peptidoglycan precursors, VanY cleaves the terminal D-alanine, generating a tetrapeptide precursor that is insensitive to the glycopeptides.

The *vanZ* gene of Tn*1546* has been shown to confer low-level teicoplanin resistance in the absence of the other *van* genes that are involved in pentadepsipeptide synthesis (Arthur *et al.*, 1995). Analysis of peptidoglycan precursors shows that VanZ-mediated resistance is not achieved through the alteration of D-Ala-D-Ala-terminating precursors (Arthur *et al.*, 1995). The mechanism of VanZ-mediated resistance has yet to be established.

### 1.7.5 Homology of Tn*1546* with other glycopeptide-resistance determinants

On the basis of the relatedness of their *van* operons, two distinct phylogenetically-related groups of glycopeptide resistance phenotypes have been identified (Abadio Patino *et al.*, 2000). The VanA, VanB and VanD phenotypes form one group, whilst VanC and VanE form the second. The glycopeptide resistance loci identified to date in enterococci are compared in figure 1.4.

## Figure 1.4: Comparison of glycopeptide resistance loci in enterococci




Colouring indicates those genes with similar functions between the gene clusters. Un-coloured arrows indicates that the genes are of unknown function. The $vanY_{G1}$ and $vanY_{G2}$ genes encode two putative D,D-peptidases that may be similar to the bifunctional enzymes of the *vanC* and *vanE* gene clusters. "% Identity" indicates amino acid identity with the homologous proteins encoded by the specified gene cluster (*vanA* or *vanC*). $vanR_E$ has only been partially sequenced.

Figures adapted from Woodford (1998), Casadewall & Courvalin (1999), McKessar *et al.* (2000) & Abadio Patino *et al.* (2000)

There is significant homology between the *vanA*, *vanB* and *vanD* gene clusters, and the proteins that they encode, particularly within the essential genes/gene products (*vanHAX* and homologues). The *vanZ* and *vanW* genes are unique to the *vanA* and *vanB* gene clusters respectively. The $vanY_D$ gene of the *vanD* gene cluster encodes a penicillin-sensitive carboxypeptidase enzyme with greater homology to catalytic serine PBPs (26%) than to the penicillin-insensitive carboxypeptidases encoded by the *vanA* and *vanB* gene clusters.

Given the relationship between the VanC and VanE phenotypes and their operons, it has been proposed that the VanE-type resistance seen in *E. faecalis* is due to the acquisition of the chromosomal *vanC* operon found in other enterococcal species (Abadio Patino *et al.*, 2000; Fines *et al*, 1999). However, the gene clusters differ significantly in their GC content, and in the amino acid identity of their encoded proteins (Abadio Patino *et al.*, 2000). This suggests that the *vanE* operon has not been acquired directly from the intrinsically-resistant *Enterococcus* species.

Based on the racemase gene ($vanT_G$) and the putative bifunctional peptidase enzymes ($vanY_{G1}$ and $vanY_{G2}$) encoded within the *vanG* locus, it may be that the VanG phenotype is related to the VanC and VanE phenotypes. Both the *vanC* and *vanE* gene clusters encode a serine racemase ($vanT_C$ and $vanT_E$) and a bifunctional enzyme with dipeptidase and carboxypeptidase activities ($vanXY_C$ and $vanXY_E$).

### 1.7.6 The origin of glycopeptide resistance genes

In the case of VanA and VanB-type resistance, the D-Ala-D-Lac terminating precursors are synthesized by ligase enzymes encoded by the *vanA* and *vanB* genes

respectively. Several studies have therefore concentrated on trying to identify *vanA* and *vanB* homologues in an attempt to identify the origin of these genes.

Lack of homology between the *vanA* or *vanB* genes and DNA from glycopeptide-sensitive enterococci suggested the resistance genes did not originate from the *Enterococcus* (Arthur *et al.*, 1993; Dutka-Malen *et al.*, 1990a; Evers *et al.*, 1994). Early studies with *vanA* and *vanB* probes also failed to identify a possible origin for the genes, the probes failing to hybridize with DNA from organisms intrinsically resistant to the glycopeptides (*Leuconostoc* spp., *Lactobacillus* spp., and *Pediococcus* spp.) or from the glycopeptide-producing organisms *Amycolatopsis orientalis* and *Actinoplanes teichomyceticus* (Dutka-Malen *et al.*, 1990a). Whilst these studies suggest the inherent resistance of the glycopeptide-producing organisms is by a different mechanism from that seen in clinical GRE, a more recent study has suggested an evolutionary relationship between the two mechanisms.

Using degenerate primers, Marshall and colleagues amplified intragenic fragments of genes likely to encode the D-Ala-D-Ala ligases (*ddl* genes) of glycopeptide-producing organisms (Marshall *et al.*, 1997). Sequence comparison of the *ddl* PCR products obtained with the sequences of the *vanA* and *vanB* genes showed the *ddl* genes of the glycopeptide-producing organisms to have a significantly higher GC content than the *vanA* or *vanB* genes. This is consistent with there having been no recent direct transfer from the antibiotic-producers to enterococci. However, comparison of the predicted amino acid sequences of the D-Ala-D-Lac ligases of glycopeptide-producing organisms showed significant homology to the amino acid sequences of the VanA and VanB ligases (>60%). In contrast, the ligases of glycopeptide-producing organisms had low homology to other D-Ala-D-X ligases. It

was therefore proposed that the *vanA* and *vanB* resistance genes might have transferred from a related antibiotic producer, or via at least one intermediate species.

Recently Marshall and colleagues have also identified VanH and VanX homologues in glycopeptide-producing organisms, further supporting the hypothesis that clinical glycopeptide resistance in enterococci originated in the glycopeptide-producing organisms (Marshall *et al.*, 1998). VanH and VanX are two proteins that are essential for glycopeptide resistance in VanA GRE (see section 1.7.3). The VanH homologues found in glycopeptide-producing organisms have 54-61% amino acid homology with the VanH protein of clinical GRE, whilst the VanX homologues have 61-64% homology with VanX. Significantly, the orientation of the homologous gene clusters identified in the glycopeptide-producing organisms is identical to that seen in GRE, and the overlap of the *vanH* and *vanA* genes is conserved (Marshall *et al.*, 1998). Recently, *vanR* and *vanS* homologues have also been identified within the glycopeptide-producing organisms (Neu &Wright, 2000).

As outlined in section 1.6, a vancomycin resistance gene cluster has been identified within the biopesticide organism, *Paenibacillus popilliae* (Patel *et al.*, 2000). Five genes encoding homologues of VanY, VanZ, VanH, VanA and VanX were identified. On the basis of amino acid sequences of the homologous proteins, and the G+C contents of the genes, Patel *et al.* (2000) concluded that the resistance gene cluster in *P. popilliae* is more similar to that in GRE than are the gene clusters in the glycopeptide-producing organisms. They concluded that the genes in *P. popilliae* may have been a precursor to the vancomycin resistance genes in enterococci.

Wherever the resistance genes originated, it is clear that such a complex resistance mechanism will have taken a considerable length of time to evolve into the form now apparent in clinical isolates of enterococci. The genes found on the VanA resistance transposon Tn*1546* (see section 1.7) differ significantly from each other in their GC content (Arthur *et al.*, 1993), suggesting the genes may have different origins. It is therefore unsurprising that the emergence of acquired glycopeptide resistance in the clinics came after 30 years of vancomycin usage.

## 1.8 The impact of glycopeptide resistance in enterococci

The prevalence of glycopeptide resistance amongst clinical isolates of enterococci increased 20-fold over a four-year period from 1989-1993 (Edmond *et al.*, 1995). This was mostly attributable to increasing prevalence amongst enterococci isolated from intensive care units. With this increase continuing, many studies have compared the clinical outcomes of infections caused by glycopeptide-resistant and glycopeptide-sensitive enterococci.

### 1.8.1 Hospitalization and mortality rates

Many studies report that patients with vancomycin-resistant enterococcal bacteraemia have significantly longer hospital stays than those with vancomycin-sensitive enterococcal bacteraemia (Lucas *et al.*, 1998; Mainous *et al.*, 1997). However, it is not always clear how much of this lengthened hospitalization is due to longer therapy following onset of bacteraemic infection, as opposed to simply the length of time prior to onset of bacteraemia. Prolonged hospitalization is well recognized as a primary risk factor for enterococcal infection (Edmond *et al.*, 1995).

Likewise, comparisons of mortality rates attributable to vancomycin-resistant and vancomycin-sensitive infections are often problematic. Most studies have concluded that vancomycin resistance is not associated with increased mortality (Lucas *et al.*, 1998; Mainous *et al.*, 1997) although Linden *et al.* report otherwise, finding significantly higher mortality rates amongst patients with bacteraemia due to vancomycin-resistant *E. faecium* than amongst those infected with vancomycin-sensitive *E. faecium* (Linden *et al.*, 1996). Shortcomings of such studies frequently include the failure to adequately take into account the severity of illness, and the fact that whilst the majority of cases of vancomycin-resistant infection will be caused by *E. faecium*, the majority of vancomycin-sensitive infections will be caused by *E. faecalis*. Direct comparisons are thus difficult to make.

### 1.8.2 Therapeutic options for infections caused by GRE

One implication of glycopeptide resistance in enterococci is that there may be no therapeutic agents commercially available and/or of established efficacy for patients with serious infection caused by multi-resistant *E. faecium*. Successful therapy currently depends on the antibiotic resistance profile of the particular infecting strain. Whilst it is sometimes possible to treat infections caused by GRE with 'traditional' antibiotics such as chloramphenicol (Norris *et al.*, 1995), infections sometimes require treatment with experimental antibiotics or unproven antibiotic combinations. This has led to the rapid evaluation of numerous agents as potential replacements for vancomycin and teicoplanin.

### 1.8.2.i Glycopeptide derivatives

Glycopeptide resistance arises through alteration of the drug target conferring cross-resistance to all naturally occurring glycopeptides. Thus it was widely thought that the glycopeptide class of antibiotics would be an unlikely source of potential replacements to overcome the problem of vancomycin resistance (Nicas *et al.*, 1997). Furthermore, unlike the β-lactam class of antibiotics, the glycopeptide molecule has limited potential for chemical modification (Nicas *et al.*, 1997). Recent attention, however, has turned to *N*-alkylated glycopeptides with these semisynthetic derivatives displaying high potency against VanA and VanB enterococci (Nicas *et al.*, 1996). These compounds have the unusual property of being bactericidal against enterococci, as well as exhibiting good activity against a range of Gram-positive organisms (Nicas *et al.*, 1997). The mechanism for their superior activity over their parent molecules has yet to be established.

### 1.8.2.ii Quinupristin-Dalfopristin

One of the non-glycopeptide alternatives to vancomycin for which there is the most clinical experience is quinupristin-dalfopristin, known as Synercid™, an antibiotic of the streptogramin class (Nicas *et al.*, 1997; Pechere, 1996). Synercid, licensed in late 1999, has received particular attention owing to its *in vitro* activity against a range of glycopeptide-resistant Gram-positive organisms, including VanA and VanB *E. faecium* (Collins *et al.*, 1993). Its clinical use may be limited, however, by its lack of activity against the predominant enterococcal species *E. faecalis* and its lack of bactericidal activity against *E. faecium* (Nicas *et al.*, 1997). Emergence of resistance to the quinupristin-dalfopristin combination during Synercid therapy of *E.*

*faecium* infection has been reported (Chow *et al.*, 1997c), as has *E. faecalis* superinfection (Chow *et al.*, 1997b). Despite these problems, it is still thought likely that Synercid will prove a useful antibiotic for the treatment of infections caused by glycopeptide-resistant *E. faecium*.

### 1.8.2.iii Glycylcyclines

Whilst tetracycline resistance is common amongst enterococcal isolates, partly due to the widespread dissemination of Tn*916*-borne *tetM*, interest has focussed on semisynthetic tetracycline derivatives which may have activity against those isolates resistant to the parent compounds (Nicas *et al.*, 1997). *N,N*-dimethylglycylamido derivatives, referred to as glycylcyclines, have shown good activity against Gram-positive bacteria, including strains of multi-drug resistant *E. faecium* (Eliopoulos *et al.*, 1994). These semisynthetic derivatives are poor substrates for the transporters mediating drug efflux and show greater affinity for ribosomes than the parent compounds, hence their activity against tetracycline and minocycline-resistant strains (Bergeron *et al.*, 1996; Someya *et al.*, 1995). Like the parent compounds, however, the glycylcyclines are only bacteriostatic.

### 1.8.2.iv Fluoroquinolones

A large number of quinolones with enhanced activity against GRE, including trovafloxacin and clinafloxacin, are undergoing development (Gootz & Brighty, 1996; Nicas *et al.*, 1997). These newer compounds generally have much improved activity against enterococci than earlier class members, such as ciprofloxacin or ofloxacin (Cohen *et al.*, 1995), and their bactericidal activity suggests they may be of

use in the treatment of systemic enterococcal infections. Mutation studies with these newer fluoroquinolones have, however, demonstrated comparable mutation rates with those obtained with ciprofloxacin (Cohen *et al.*, 1995) and thus their therapeutic potential may be short-lived through the rapid acquisition of resistance.

### 1.8.2.v Oxazolidinones

Oxazolidinones are novel synthetic, broad-spectrum, bacteriostatic agents that inhibit protein synthesis (Nicas *et al.*, 1997). They were initially investigated in the late 1980s but recent attention has focussed on novel fluorinated oxazolidinones that demonstrate bactericidal activity against Gram-positive organisms, anaerobes and some Gram-negative species (Jones *et al.*, 1996). No cross resistance to other antibiotic classes has yet been described and it is thought that these compounds show potential for the treatment of infections caused by various organisms, including GRE (Nicas *et al.*, 1997).

### 1.8.3 The threat of dissemination of vancomycin resistance genes

Arguably the main concern that was raised by the emergence of glycopeptide resistance in enterococci was the threat of its transfer to other organisms, particularly the 'first-rate' pathogens such as MRSA or penicillin-resistant pneumococci. The potential for the spread of the glycopeptide resistance genes has already been illustrated, with *vanA* being identified in various bacterial species, although not necessarily associated with Tn*1546*-like elements (French *et al.*, 1992; Ligozzi *et al.*, 1998; Power *et al.*, 1995). However, despite MRSA and GRE coexisting in the

nosocomial setting for over a decade, transfer into *S. aureus* in the clinical setting has not been detected.

Noble and colleagues have demonstrated the transfer of *vanA* into *S. aureus* under laboratory conditions (Noble *et al.*, 1992), although the strains used were not MRSA. In the clinical setting MRSA appear to develop resistance to the glycopeptides by an alternative method: that of augmented cell wall production and PBP expression (Hanaki *et al.*, 1998). Such MRSA strains exhibiting reduced susceptibility to vancomycin were first reported in Japan (Hiramatsu *et al.*, 1997), and subsequently in Europe and the USA (CDC, 1997; Howe *et al.*, 1998; Ploy *et al.*, 1998), although they remain rare. Their clinical significance remains uncertain, as doubling the thickness of the bacterial cell wall is unlikely to be an efficient resistance mechanism, and the levels of glycopeptide resistance conferred are significantly lower than those mediated by VanA in enterococci.

Finally, it is interesting to speculate that *vanA*-mediated glycopeptide resistance will not arise in MRSA due to incompatibility of the two resistance mechanisms. The methicillin resistance mechanism of MRSA and glycopeptide resistance conferred by the *vanA* gene cluster arise through altering either the structure of the cell wall or components involved in cell wall synthesis. It is possible, therefore, that the lack of *vanA*-mediated glycopeptide resistance in MRSA is due to the inability of the two resistance mechanisms to coexist within the bacterial cell.

### 1.9 Epidemiology of glycopeptide-resistant enterococci

Reports that followed the initial identification of acquired glycopeptide resistance in enterococci in the late 1980s were consistent with the glycopeptide resistance trait

being another example of a nosocomial resistance trait, similar in many respects to methicillin resistance in *S. aureus*. Glycopeptide resistance was found predominantly, if not exclusively, in the nosocomial setting (Murray, 1997). However, European studies in the 1990s began to reveal a significant reservoir of GRE in various non-hospital sources and it is now recognized that the epidemiology of GRE is extremely complex and potentially influenced by several factors.

### 1.9.1 The European-American paradox

GRE have become a major infection control problem in the United States, far more so than in Europe (Woodford, 1998). Despite their prevalence in US hospitals, no GRE have been isolated from community or non-human sources within the States (Coque *et al.*, 1996), with the exception of one VanA GRE isolated from a sample of dry dog food (Dunne *et al.*, 1996) and one multi-resistant VanB GRE from animal feed (Schwalbe *et al.*, 1999). This is in stark contrast to the situation now being reported in many European countries. Whilst the scale of the problem of nosocomial GRE in Europe is not at the level being witnessed in the States, within Europe GRE are isolated from a variety of non-hospital sources including the community, pet and farm animals, raw meat and sewage (Aarestrup *et al.*, 1996; Bates *et al.*,1994; Devriese *et al.*, 1996; Klare *et al.*, 1995a).

A study in Belgium by van der Auwera and colleagues reported the recovery of GRE from 28% of community-based volunteers with no known hospital or glycopeptide exposure, the isolates being primarily polyclonal VanA (van der Auwera *et al.*, 1996). In the same report they describe the isolation of GRE from 64% of healthy volunteers who had been administered oral vancomycin or

teicoplanin (van der Auwera *et al.*, 1996). No GRE were detected in the faecal flora prior to glycopeptide administration. As acquired glycopeptide resistance in enterococci does not arise through spontaneous mutation, these findings suggest that, in Europe at least, GRE are frequent colonizers of the GI tract of healthy, non-hospitalized individuals, albeit in low numbers. These GRE may only reach detectable levels following glycopeptide therapy. It is therefore possible that GRE observed in the nosocomial setting within Europe actually have a community origin and are only seen following the intense selective pressures encountered in hospitals.

With the isolation of GRE from uncooked chickens and minced-meat products in Europe (Bates *et al.*, 1993; Howarth & Poulter, 1996), it seems such meat and poultry products may act as a source for these community-based GRE. The ruggedness and heat tolerance of enterococci would be expected to enhance their ability for survival once having entered the food chain.

### 1.9.2 GRE in animal husbandry

#### 1.9.2.i *E. faecium* as an animal food supplement

A potential source of GRE in animal livestock that has received little attention is the use of *E. faecium* as an animal food supplement (Murray, 1997). *E. faecium* has a long history of use, being fed to livestock to stabilize and modulate the intestinal flora. The rationale behind its use is that *E. faecium* will confer colonization resistance, lactic acid and possibly bacteriocin production by *E. faecium* acting as a barrier to pathogenic bacteria. Ironically, it is the very multi-drug resistant nature of *E. faecium* that has made it a popular candidate for use as a food supplement. It is possible that GRE were amongst those *E. faecium* strains used as a food supplement.

Prior to the initial description of enterococci with acquired glycopeptide resistance, the routine testing of clinical enterococcal isolates for this resistance trait was not widespread. It is possible, therefore, that *E. faecium* strains destined for use as a food supplement were not screened for glycopeptide resistance.

### 1.9.2.ii GRE and avoparcin

In the late 1960s, a report was commissioned to examine the use of antibiotics in animal husbandry and veterinary medicine (HMSO, 1969). Amongst the recommendations of the report was that non-prescription use of antibiotics in animal feed should be restricted to those antibiotics which would not impair the efficacy of therapeutic antibiotics through the development of resistance (HMSO, 1969). Despite this recommendation, the glycopeptide antibiotic avoparcin went into use as a growth-promoting agent in animal husbandry in 1975, even though resistance to avoparcin confers cross-resistance to vancomycin and teicoplanin.

Antibiotic growth promoters have been used in animal husbandry since the 1940s, particularly in the rearing of pigs and poultry (Bonner, 1997). It is well established that animals reared in a germ-free environment grow faster than those reared in conventional surroundings (Coates *et al.*, 1963). Coates *et al.* demonstrated that the growth rate of conventionally-reared animals can, however, be increased almost to that of the germ-free animals by administering low-level antibiotics continuously in their feed (Coates *et al.*, 1963). The exact mechanism by which antibiotics improve growth and feed conversion efficiency is unknown, but it is thought that the subtherapeutic levels administered are able to control "low-level" diseases which would otherwise stunt the growth of the animals (Bonner, 1997). All effects are

limited to the intestinal bacteria, as the agents used are administered orally and are poorly absorbed.

Whilst it has been proposed that the low-level use of antibiotics as growth-promoters does not select resistant strains amongst the intestinal flora (Smith, 1993), there is increasing evidence to suggest otherwise. In Denmark, Aarestrup and colleagues reported high levels of vancomycin and avoparcin resistance amongst faecal isolates from animals (Aarestrup *et al.*, 1996). As vancomycin is not used therapeutically in veterinary medicine, they concluded that the use of avoparcin was responsible for the selection of GRE. Aarestrup *et al.* also proposed that, owing to the passage of avoparcin through the digestive system both largely unabsorbed and unmetabolized, the concentration of antibiotic in the GI tract is probably in excess of the avoparcin MIC for susceptible enterococci (Aarestrup *et al.*,1996). Several studies have now reported a correlation between the use of avoparcin for growth promotion and isolation of VanA GRE from farm animals (Aarestrup, 1995; Klare *et al.*, 1995b). In the face of such findings, the use of avoparcin in the European Union was banned in 1997, owing to the potential risk it posed to human health through the selection of GRE.

The use of growth promoters has long been recognized as having economic advantages (Bonner, 1997; MacKinnon, 1993) and it was therefore considered likely that alternative antimicrobial growth-promoting agents would be used in place of avoparcin following its withdrawal (Woodford, 1998). If resistance to these alternative antimicrobials is linked to glycopeptide resistance genes, it could be anticipated their use would continue to select GRE following the withdrawal of avoparcin (Woodford, 1998). Early indications are, however, that avoparcin

withdrawal has led to a decreased incidence of GRE in animal livestock (Aarestrup *et al.*, 2000; Klare *et al.*, 1999b) and, perhaps more surprisingly, the complete withdrawal of antimicrobial growth promoters in poultry production was not associated with economic loss (Emborg *et al.*, 1999).

Based on the above, there has certainly been the potential for GRE to be introduced and/or selected in animal livestock, with these GRE subsequently entering the food chain through the contamination of meat products. Following ingestion, these GRE strains may colonize the human gut or transfer their resistance determinants to the endogenous enterococcal flora. The relative importance of avoparcin use versus the clinical use of vancomycin and teicoplanin as factors promoting the selection and dissemination of GRE is still unclear. In the United States avoparcin has never been licensed for use, a fact which may at least partly explain the lack of GRE in community and non-human sources in the US. The problem of GRE in the US is most likely due to the use and abuse of vancomycin in the clinics. The contrasting picture in Europe suggests avoparcin was certainly implicated to an extent in the widespread dissemination of GRE, but the clinical significance of its use remains uncertain.

### 1.9.3 Epidemiology of nosocomial GRE

#### 1.9.3.i Diversity of nosocomial GRE

On the basis of pulsed-field gel electrophoresis (PFGE) analysis and their isolation from at least two patients in at least two hospitals, five epidemic strains of vancomycin-resistant *E. faecium* (designated EVREM) have been defined (Woodford, 1998). Of these five strains, EVREM-3 is causing the greatest concern,

being isolated from many hospitals in the London area. EVREM-3 exhibits high-level resistance to the aminoglycosides, penicillins, and various other antibiotics, as well as VanA glycopeptide resistance.

With the exception of these epidemic strains, GRE isolated from hospitals within the UK are generally very diverse, with PFGE analysis revealing considerable heterogeneity, both within individual hospitals and between different hospitals (Woodford, 1998). This is likely to be due, at least in part, to the transposable nature of the *vanA* gene cluster and the frequent association of Tn*1546* with transmissible plasmids. This association will have aided the widespread dissemination of VanA glycopeptide resistance to diverse strains of enterococci.

Outbreaks of GRE infection usually occur within specialized units, including renal, haematological and intensive care units (Murray, 1997; Woodford, 1998). Whilst some hospitals may report a predominant strain of GRE, responsible for such outbreaks, predominant strains from different hospitals usually differ, and sporadic isolates tend to be unique by PFGE (Woodford, 1998). This is once again in contrast to the epidemiology of GRE in the US, where hospitals frequently report the predominance of a single strain (Murray, 1997). Evidence suggests that GRE isolated in hospitals across the UK have emerged independently at these different centres, consistent with their endemicity in the community and their subsequent selection following antibiotic therapy in the hospital setting (Woodford, 1998).

### 1.9.3.ii Prevalence of VanA versus VanB phenotypes

Of the GRE referred from hospitals in England and Wales to the PHLS Laboratory of Hospital Infection in the period from 1987 to 1996, 87.5% were VanA and 12.5%

VanB (Morrison *et al.*, 1997). Whilst low detection rates of VanB isolates owing to their sometimes borderline-levels of resistance may partly explain the discrepancy between the two phenotypes, it is unlikely to be the sole reason. Although interest in the VanB phenotype and the apparent differences in the prevalence of the VanA and VanB phenotypes have led to studies being more rigorous in their detection systems for VanB enterococci, the discrepancy remains. Numerous studies addressing the prevalence of GRE in the community and from non-human sources have failed to demonstrate the presence of VanB phenotype enterococci outwith the hospital setting (Woodford, 1998). Globally there is only one, unsubstantiated, report of a VanB *Enterococcus* isolated from a non-human source, being isolated from a cow in Australia in 1997 (John Ferguson; personal communication). The *vanB* gene has also been identified in *Streptococcus gallolyticus*, isolated from veal calves in The Netherlands (Mevius *et al.*, 1998)

Differing prevalence of VanA and VanB phenotypes may then be partly explained by there being a significant environmental reservoir of VanA enterococci, but no VanB equivalent. It is likely, however, that other factors will also play a role. It may be that the predominance of VanA phenotype is due to the frequent association of the *vanA* gene cluster with plasmids and occasionally conjugative transposons that have facilitated its widespread dissemination. VanB phenotype was long regarded as being chromosomal, and although it has now been associated with many conjugative transposons and plasmids, they may not be providing as efficient dissemination as the *vanA*-bearing plasmids. Similar reasons have been used to explain the predominance of TEM-1 β-lactamase over TEM-2. The use of teicoplanin in hospitals would also be expected to promote the selection of VanA phenotype over

VanB, the latter phenotype not displaying teicoplanin resistance. However, the level of teicoplanin usage is generally far lower than that of vancomycin and so it is unlikely to be a major factor in the discrepancy between the two phenotypes.

### 1.9.3.iii Relatedness of GRE from human and non-human sources

With the controversy surrounding the use and subsequent withdrawal of avoparcin as a growth-promoting agent in animal livestock and the potential consequences for both the animal husbandry and pharmaceutical industries, molecular studies are necessary to determine the relatedness of VanA GRE from human and non-human sources. However, with such great diversity amongst hospital isolates of GRE, conclusive evidence for strains common to man and other sources (particularly animals) is scarce. PFGE and ribotyping are commonly applied, but the horizontal transfer of resistance genes mediated by both plasmids and transposons leads to genetically diverse GRE populations. Only in a limited number of cases have isolates sharing PFGE or ribotype patterns been demonstrated in both humans and animals or animal-products. In one study, two clinical isolates from separate hospital patients shared ribotype patterns with porcine isolates (Bates *et al.*, 1994), whilst another study reported isolates with similar PFGE patterns from humans and minced meat (Klare *et al.*,1995b). Identical GRE isolates have also been reported in turkeys and their farmer (van den Bogaard *et al.*, 1997).

With such heterogeneity amongst GRE isolates pointing to the dissemination of Tn*1546* by plasmid transfer, interest has recently turned to investigating the diversity of the Tn*1546*-related elements themselves.

### 1.9.4 Diversity of Tn*1546*-related elements

Whilst the overall structure of the *vanA* gene cluster is generally conserved, the genetic organization being as shown in figure 1.3, there is now known to be considerable diversity amongst the Tn*1546*-related elements present in VanA GRE from both human and non-human sources. Variation within Tn*1546*-related elements, which is primarily found in the regions upstream from *vanR* and downstream from *vanX*, is generally attributable to point mutations and the presence of insertion sequences (IS elements) and deletions, with some studies also taking into account the integration position of Tn*1546* (Handwerger *et al.*, 1995; Jensen *et al.*, 1998; Palepou *et al.*, 1998; Willems *et al.*, 1999; Woodford *et al.*, 1998). Several IS elements have now been identified within Tn*1546*-related elements, including IS*1542*, IS*1251*, IS*1476* and IS*1216V*-related elements (Darini *et al.*, 1999; Handwerger *et al.*, 1995; MacKinnon *et al.*, 1997; Willems *et al.*, 1999; Woodford *et al.*, 1998). Deletion events often accompany these insertions and are frequently located in the *orf1*/*orf2* region (Palepou *et al.*, 1998; Willems *et al.*, 1999).

Identical transposon types have been identified in VanA GRE isolated from both human and non-human sources (Jensen *et al.*, 1998; Willems *et al.*, 1999; Woodford *et al.*, 1998), suggesting a common pool of glycopeptide resistance determinants. However, the significance of such findings has been questioned, owing to the potential instability of the variation being described, particularly that attributable to the presence of IS elements. Often Tn*1546* types differ only by one insertion event and the IS elements responsible are frequently found elsewhere within the enterococcal genome. Therefore the assigned Tn*1546* type can be altered by a single transposition event. Such Tn*1546* instability has been reported, both within the

clinical setting and during prolonged cold storage of VanA GRE (Darini *et al.*, 2000; Woodford *et al.*, 1998).

Thus questions remain over the validity of Tn*1546* analysis as an epidemiological tool. However, it is likely to remain a useful marker, even if it is restricted to 'snap-shot' pictures of epidemiology. Interestingly, the restricted geographical distribution of certain IS elements may be of use as epidemiological markers. In 1995, IS*1251* was identified within the *vanS-vanH* intergenic region of Tn*1546*-related elements in VanA GRE from the United States (Handwerger *et al.*, 1995). Only recently has this alteration within the *vanS-vanH* intergenic region been identified in European GRE isolates, suggesting recent intercontinental spread (Simonsen *et al.*, 2000).

# Aims of this thesis

- To establish the *van* genotype of glycopeptide-resistant enterococci in Scottish hospitals.

- To determine the heterogeneity of nosocomial GRE by PFGE analysis, and to compare those GRE strains with GSE isolates from hospital and community sources.

- To study VanA GRE isolates for the ability to transfer *vanA*-mediated glycopeptide resistance, and to establish the nature of the element responsible.

- To examine the diversity of Tn*1546*-related elements amongst VanA GRE in Scotland, and to assess any impact the variation is having on the expression of glycopeptide resistance.

# Chapter 2: Materials & Methods

## 2.1 Bacterial strains

Glycopeptide-resistant enterococci were collected from eight Scottish hospitals over a five-year period (1995-1999) following their referral by the hospital's clinical diagnostic laboratories. In addition, a five-month survey of enterococcal epidemiology was undertaken, during which time, enterococcal isolates were collected from the diagnostic laboratories of the Royal Infirmary of Edinburgh (see section 2.3.1).

Enterococci suspected of being glycopeptide-resistant were subjected to vancomycin and teicoplanin susceptibility testing (see section 2.3.2) to confirm their glycopeptide resistance phenotype. Identification of isolates to the species level was performed by the API20 Strep System (BioMerieux, Marcy-l'Etoile, France) according to the manufacturer's instructions.

Table 2.1 lists the standard bacterial strains used in the study as control organisms.

**Table 2.1: Standard bacterial strains**

| Bacterial strain | Resistance | Reference |
|---|---|---|
| *E. faecium* NCTC 12202 | VanA phenotype | Uttley *et al.* (1988) |
| *E. faecalis* ATCC 51299 | VanB phenotype | Swenson *et al.* (1995) |
| *E. gallinarum* NCTC 12359 | VanC-1 phenotype | Collins *et al.* (1984) |
| *E. casseliflavus* NCTC 12361 | VanC-2 phenotype | Collins *et al.* (1984) |

Bacterial strains were stored at -70°C in Brain-Heart Infusion (BHI) broth supplemented with glycerol to 5% v/v. Subculture of stored strains was performed on BHI agar plates with a vancomycin disc to monitor stability of glycopeptide resistance.

## 2.2 Materials

### 2.2.1 Media

Complex media used were BHI broth and agar, Isosensitest agar (ISTA), Mueller-Hinton agar (MHA) and Kanamycin-Aesculin-Azide agar base (KAA). All were supplied by Oxoid (Basingstoke, UK), and prepared according to the manufacturer's instructions. Media were sterilized by autoclaving at 121°C / 15 psi for 15 minutes. All cultures were prepared in BHI medium unless stated otherwise.

### 2.2.2 Reagents

All chemicals and reagents were supplied by Sigma-Aldrich Company Ltd. (Poole, UK) unless otherwise stated.

### 2.2.3 Antimicrobial agents

Antimicrobial agents used in this study are listed in table 2.2. All were stored at 4°C, with stock solutions freshly prepared as required. Antibiotics were dissolved in sterile ultra-pure deionized water alone, or in combination with absolute alcohol.

**Table 2.2: Antimicrobial agents**

| Antimicrobial | Supplier / Manufacturer |
|---|---|
| Vancomycin | Eli Lilly, Basingstoke, UK |
| Teicoplanin | Marion Merrell, Uxbridge, UK |
| Avoparcin | Hoffman-la-Roche, Basle, Switzerland |
| Erythromycin | David Bull Laboratories, Victoria, Australia |
| Ampicillin | Sigma-Aldrich Company Ltd. |
| Tetracycline | Sigma-Aldrich Company Ltd. |
| Chloramphenicol | Sigma-Aldrich Company Ltd. |
| Streptomycin | Sigma-Aldrich Company Ltd. |
| Gentamicin | Sigma-Aldrich Company Ltd. |
| Rifampicin | Marion Merrell |
| Fusidic Acid | Sigma-Aldrich Company Ltd. |

### 2.2.4 Oligonucleotide primers

All primers were synthesized by Oswel DNA Services Ltd., Southampton. Those for sequencing were HPLC purified

## 2.3 Methods

### 2.3.1 Survey of enterococcal epidemiology

Whilst the primary objective of this thesis is to assess the diversity of VanA phenotype GRE in Scotland, it was considered beneficial to study the wider issue of enterococcal epidemiology, and not simply those isolates displaying glycopeptide resistance. This enables comparisons to be made between the glycopeptide-resistant and sensitive enterococcal populations present in hospital and community settings.

To achieve this, a five-month survey of enterococcal epidemiology was undertaken (January-May, 1998). During this period, enterococcal isolates were collected from the diagnostic laboratories of the Royal Infirmary of Edinburgh, both hospital and general practice isolates. In addition, enterococci were isolated from a cross-section of faecal samples submitted to the diagnostic laboratories for investigation. Isolation was achieved with selective media comprising of kanamycin-aesculin-azide agar base, supplemented with 15mg/L nalidixic acid and 10mg/L colistin sulphate (Staph/Strep Selective Supplement, Oxoid). Aesculin hydrolysis by enterococci results in black colouration of the agar, allowing tentative identification as an *Enterococcus*. One presumed *Enterococcus* was taken from each faecal sample for further investigation.

All enterococcal isolates gathered during this five-month period were screened for vancomycin resistance with 5μg vancomycin discs (Becton Dickinson & Co., Cockeysville, USA). Any isolates tentatively identified as GRE were subjected to full glycopeptide susceptibility testing and PCR analysis (sections 2.3.2 and 2.3.3). All isolates were speciated by the API20 Strep System and subjected to PFGE analysis, as described in section 2.3.5.

### 2.3.2 Antimicrobial susceptibility testing

For routine antimicrobial sensitivity testing, the tests were performed on MHA plates with minimum inhibitory concentrations (MICs) determined by agar incorporation of the antimicrobial agents (British Society for Antimicrobial Chemotherapy Working Party, 1991). Antibiotic stock solutions were prepared freshly at concentrations permitting the preparation of two-fold dilutions of antibiotic in MHA. Agar was allowed to cool to 50°C prior to addition of the antibiotic and, once set, the MIC plates were allowed to dry thoroughly, either at room temperature or for 15 minutes at 55°C.

Bacterial strains were subcultured from -70°C storage on BHI agar plates prior to inoculation into BHI broth. Following overnight incubation in an orbital shaker at 37°C, a 100-fold dilution of the overnight culture was made in sterile saline (0.85% sodium chloride). These diluted cultures were then used to inoculate the MIC plates with a Denley multi-point inoculator (Denley, Billinghurst, Surrey), resulting in approximately $10^4$ CFU/spot. After allowing the inocula to dry, plates were incubated at 37°C for 18-24 hours. The MIC was defined as the lowest concentration of antibiotic to inhibit all visible growth. The $MIC_{50}$ and $MIC_{90}$ (concentration of antibiotic required to inhibit 50% and 90% of bacterial strains respectively) were determined when appropriate.

In an attempt to determine the basis of a media-dependent increase in teicoplanin resistance associated with Tn*1546* genotype (section 6.1), teicoplanin MICs were later repeated on ISTA supplemented with either pyruvate (0.1mM or 0.01mM final concentrations) or D,L-lactic acid (0.1% or 0.01% final concentrations). The protocol

did not vary from that described above, other than the inclusion of pyruvate or lactic acid within the agar.

### 2.3.3 Detection of glycopeptide resistance genes by PCR

Determination of the vancomycin and teicoplanin MICs of the strains collected allowed a prediction to be made as to their glycopeptide resistance phenotype on the basis of their levels of resistance to the two glycopeptide agents. All isolates exhibiting high-level resistance to vancomycin and teicoplanin were presumed to be VanA-type, whilst those displaying only vancomycin-resistance were presumed to be VanB. These predicted phenotypes were confirmed by PCR detection of the glycopeptide resistance genes with the *vanA*- and *vanB*-specific primers described by Dutka-Malen and colleagues. (Dutka-Malen *et al.*, 1995). Primer sequences are shown in table 2.3.

*E. faecium* NCTC 12202 and *E. faecalis* ATCC 51299 were used as *vanA* and *vanB*-positive controls respectively. *E. faecalis* JH2-2 (Jacob & Hobbs, 1974) was used as a negative control.

**Table 2.3: Primers for detection of *vanA* and *vanB* genes**

| Primer | Sequence (5'-3') | Coordinates |
|---|---|---|
| *vanA*1 | (+) GGGAAAACGACAATTGC | 7154-7170[a] |
| *vanA*2 | (−) GTACAATGCGGCCGTTA | 7885-7869[a] |
| *vanB*1 | (+) ATGGGAAGCCGATAGTC | 5144-5160[b] |
| *vanB*2 | (−) GATTTCGTTCCTCGACC | 5778-5762[b] |

[a] Coordinates correspond to GenBank accession number M97297

[b] Coordinates correspond to GenBank accession number U35369

Crude DNA template for the PCR reaction was prepared by boiling bacterial cells (0.8mL overnight culture and 0.2mL sterile deionized water) for 10 minutes in a boiling water bath. Following centrifugation for 5 minutes to remove debris, 20µL of supernatant was used as template in the PCR reactions. PCR was performed in 100µL volumes containing 40pmol of each oligonucleotide primer, dNTPs to a final concentration of 500µM, 3mM $MgCl_2$ and 2 U of *Taq* DNA polymerase (Advanced Biotechnologies, Dorking, Surrey) with appropriately diluted manufacturer's reaction buffer. PCR was performed in a Techne Cyclogene Thermal Cycler (Cambridge) with the protocol: (i) 94°C for 3 min; (ii) 25 cycles of 94°C for 45s, 45°C for 30s, and 74°C for 90s; and (iii) 74°C for 10 min. PCR products were analysed by electrophoresis (see section 2.3.4).

### 2.3.4 Analysis of DNA by agarose gel electrophoresis

PCR products, plasmid extractions and restriction endonuclease digests were typically electrophoresed on 1.5% agarose gels in TAE buffer (40mM Tris-acetate pH 8.0; 2mM EDTA). Electrophoresis was performed on horizontal slab gels in the Bio-Rad Sub-Cell® GT agarose gel electrophoresis system. DNA samples were mixed with loading buffer (0.25% w/v bromophenol blue; 0.25% w/v xylene cyanol; 30% w/v sucrose) to a ratio of 5:1 prior to loading onto the gel. Samples were electrophoresed alongside either a 100-bp DNA ladder or GeneRuler™ DNA Ladder Mix (Helena BioSciences Ltd., UK) or a Lambda/*Hind*III DNA ladder (GibcoBRL, Life Technologies Ltd., Paisley). The choice of DNA ladder was dependent on the size of DNA fragment being analysed. Electrophoresis was performed at a constant voltage of 100V until the loading buffer fronts had moved two-thirds of the way

down the gel. Following electrophoresis, gels were stained in 0.5μg/mL ethidium bromide and visualized on a UV transilluminator. Photographs were taken with a Polaroid camera system fitted with an orange filter.

### 2.3.5 Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was applied to assess the relatedness of isolates. Overnight cultures (10mL) were harvested by centrifugation in a Sorvall RT6000D bench-top centrifuge (3000rpm; 10 minutes) prior to washing and resuspension in PIV buffer [1M NaCl, 10mM Tris-HCl (pH 7.6)]. To ensure reproducibility of results, the cell suspension was standardized with PIV to an optical density of 1.0 at 610nm in a Perkin-Elmer Lambda 2 spectrophotometer (Beaconsfield, Bucks.). An equal volume of the standardized cell suspension was mixed with 1.6% low-melting preparative grade agarose (Bio-Rad Laboratories Ltd., Herts) prepared in lysis buffer [6mM Tris-HCl (pH 7.6), 1M NaCl, 100mM EDTA (pH 7.5), 0.5% Brij 58, 0.2% deoxycholate and 0.5% sodium lauroyl sarcosine] and pipetted into plug moulds.

The protocol then followed that described by Miranda and colleagues (Miranda *et al.*, 1991). Briefly, the cells embedded in the agarose plugs were lysed by overnight incubation at 37°C in lysis buffer, supplemented with 20μg/mL RNase and 1mg/ml lysozyme. Plugs were then incubated overnight at 50°C in ESP solution [0.5M EDTA (pH 9.0-9.5), 1% sodium lauroyl sarcosine, and 50μg/mL proteinase K] prior to washing with TE buffer [10mM Tris-HCl (pH 7.5), 0.1mM EDTA]. At this stage, plugs could be stored at 4°C in TE buffer.

For PFGE analysis, a slice of plug was incubated with 20 U of *Sma*I restriction endonuclease (Promega UK, Southampton) in a 200µL reaction volume for 6 hours at 25°C. The plugs were then washed in TE buffer for 1 hour before being sealed into the wells of a 1.2% agarose gel prepared in 0.5x TBE buffer (Sigma). Electrophoresis was performed on a contour-clamped homogeneous electric fields device (CHEF-DRII; Bio-Rad Laboratories Ltd.). The gel was run for 24 hours at 200V with 5-40 second pulse times. Gels were stained with ethidium bromide (0.5µg/ml) for 15 minutes, and destained in distilled water (2 x 15 minutes) prior to visualization by UV.

### 2.3.6 Interpretation of PFGE patterns

#### 2.3.6.i Visual comparison of PFGE patterns

PFGE patterns were initially compared by eye, and interpreted by the criteria described by Tenover and colleagues (Tenover *et al.*, 1995). Using these criteria, isolates are deemed to be indistinguishable if they have identical PFGE patterns. Isolates differing by up to three bands are considered to be closely related, with four to six bands difference suggesting isolates are "possibly related". Isolates are considered unrelated if they differ by seven or more bands.

Examples of the different PFGE types identified were rerun on the same gel to overcome difficulties in comparing patterns on different gels.

#### 2.3.6.ii Computer-aided comparison of PFGE patterns

It was latterly decided to assess the diversity of PFGE patterns with the Bio-Rad Diversity Database software.

Gel images were introduced into the Diversity Database from the original Polaroid photographs. Analysis was then performed according to the manufacturer's guidelines. The results of the comparisons were obtained in two forms: phylogenetic tree and similarity matrix.

### 2.3.7 Conjugation by filter mating

The protocol used for filter mating was adapted from that described by Heaton and Handwerger (Heaton & Handwerger, 1995). VanA *E. faecium* were mated with *E. faecium* GE-1 (Eliopoulos *et al.*, 1982), whilst VanA *E. faecalis* were mated with *E. faecalis* JH2-2 (Jacob & Hobbs, 1974). Both recipients (donated by Dr. N. Woodford) display resistance to rifampicin (>100mg/L) and fusidic acid (>25mg/L).

Overnight cultures of donors and recipients were diluted 100-fold in fresh broth and incubated for 2-3 hours. Ten microlitres of donor culture was then mixed with 90μL of recipient culture and the resulting 100μL dispensed onto a sterile 25mm, 0.2μm pore size filter, previously placed on a BHI agar plate. The cell suspension was allowed to 'dry' onto the filter prior to incubation at 37°C overnight.

Following incubation for 24 hours, the filter was placed in 10mL BHI broth and the cells resuspended. Selective plates containing rifampicin (100mg/L), fusidic acid (25mg/L) and vancomycin (100mg/L) were inoculated with 100μL of the mating mixture and incubated overnight. Control plates were also prepared to ensure the sensitivity of donors to rifampicin and fusidic acid, and the sensitivity of recipients to vancomycin. After incubation of selective plates for 24 hours they were examined for the presence of transconjugant colonies. In the absence of colonies, plates were reincubated for a further 24 hours.

### 2.3.8 Curing of plasmid DNA with ethidium bromide

The intercalation of ethidium bromide between base-pairs of DNA inhibits the functions of both DNA and RNA polymerase. As it preferentially inhibits plasmid synthesis, it can be used to eliminate ('cure') plasmid DNA from bacterial cells (Bouanchard *et al.*, 1969).

Ethidium bromide MICs were established for those isolates to be used in plasmid curing experiments. Ethidium bromide plates for MIC determination ranged in concentration from 5-70 mg/L. Overnight broth cultures were diluted 100-fold and used to inoculate fresh BHI broths containing increasing concentrations of ethidium bromide (2mg/L increments covering the concentration range, MIC ± 10mg/L). After overnight incubation, the broths were examined for visible growth. The broth that contained the highest concentration of ethidium bromide and still had visible growth was plated onto BHI agar plates. After overnight incubation, individual colonies from this plate were examined for loss of glycopeptide resistance that may be attributable to loss of plasmid DNA.

### 2.3.9 Extraction of plasmid DNA

Several different plasmid extraction protocols were employed in the course of this study, but the following protocol was employed most widely. Cells were harvested from 10mL overnight BHI broth cultures by centrifugation and washed in TE buffer [10mM Tris, 1mM EDTA, pH 8.0]. The protocol then followed that described by Woodford *et al.* (1993). Briefly, cells were suspended in 100µL of suspending buffer (25% sucrose in TE buffer supplemented with 10mg/mL lysozyme) and incubated at 37°C for 30 minutes. The cells were lysed by the addition of 200µL

alkaline-SDS (0.2M NaOH, 1% SDS), and the samples were incubated at 56°C for 1 hour. After addition of 150µL 3M potassium acetate (pH 4.8), the samples were left on ice for 20 minutes. Following centrifugation for 5 minutes, the precipitated debris was removed by decanting the supernatant through a double-thickness of medical gauze (Boots plc., Nottingham, UK) into a fresh tube. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and samples were centrifuged for 5 minutes. The DNA from 200µL of supernatant was precipitated with 2 volumes of cold ethanol at room temperature for 2 minutes prior to harvesting by centrifugation. DNA pellets were allowed to dry prior to resuspension in distilled water supplemented with 1µg of RNase.

Other plasmid extraction methods employed included the RPM® Spin Midi kit (BIO 101 Inc., LoJolla, California) and the large-scale plasmid extraction method described by Heilig *et al.* (1998). These methods, as with the plasmid extraction procedure described above, are based on the alkaline lysis method described by Birnboim (1983).

### 2.3.10 Detection of plasmids by electrophoresis of 'intact' genomic DNA

Crosa *et al.* (1994) described a method for the detection of plasmid DNA that is based on bacterial cell lysis directly in the wells of an agarose gel. Lysis within the wells ensures the majority of the chromosomal DNA remains intact and thus remains within the wells of the gel. Less than 0.5% of the total chromosomal DNA should appear within the gel as a band of linear DNA (Crosa *et al.*, 1994). Plasmid DNA, however, should migrate freely from the wells of the agarose gel, enabling its detection.

As an alternative to lysis within the wells of the gel, plugs previously prepared for PFGE analysis were electrophoresed unrestricted (i.e. without restriction of genomic DNA with *Sma*I restriction endonuclease). Plug sections were loaded into the wells of a horizontal slab gel and electrophoresed for approximately 4 hours at 125V. Gels were stained and visualized as described previously (section 2.3.4).

### 2.3.11 Extraction of genomic DNA

High-quality genomic DNA was extracted with guanidium thiocyanate as described by Pitcher *et al.* (1989). Briefly, 10mL overnight broth cultures were harvested by centrifugation and washed in TE buffer prior to resuspension in 100µL TE supplemented with 50mg/mL lysozyme. Following incubation at 37°C for 30 minutes, cells were lysed by the addition of 0.5mL GES reagent (5M guanidium thiocyanate, 100mM EDTA and 0.5% v/v sarkosyl). Lysates were cooled on ice prior to the addition of 0.25mL cold (-20°C) 7.5M ammonium acetate. Samples were held on ice for a further 10 minutes prior to extraction of DNA with chloroform/isoamylalcohol. DNA was precipitated by the addition of 0.54 volumes of cold (-20°C) 2-propanol and collected by centrifugation. DNA pellets were washed in 70% ethanol prior to redissolving in 100µL of sterile deionized water.

### 2.3.12 Quantitation of DNA

Rapid estimation of DNA concentration was performed by ethidium bromide dot quantitation as described by Moore and Chory (1998). Lambda/*Hind*III DNA (GibcoBRL) was used to prepare DNA standards ranging in concentration from 0 to 20µg/mL. DNA to be quantified was mixed with ethidium bromide and spotted onto

Saran wrap adjacent to the DNA standards. Comparison of fluorescence under UV illumination of the unknown DNA against the standards provided an estimation of the DNA concentration of the unknowns.

### 2.3.13 Design of primers for Tn*1546* analysis

Primers used in the analysis of Tn*1546*-related elements had either been described previously or were designed by Primer3 (http://www.genome.wi.mit.edu.cgi-bin/primer) based on the published sequence of Tn*1546* (GenBank accession number M97297; Arthur *et al.* (1993, 1995)). Primers used are listed in table 2.4.

### 2.3.14 PCR analysis of Tn*1546*-like elements

#### 2.3.14.i Long-range PCR (L-PCR) analysis of Tn*1546*-like elements

Tn*1546*-like elements were amplified with the Expand™ Long Template PCR System (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The PCR system combines *Taq* DNA polymerase with the *Pwo* DNA polymerase that has proof-reading activity. This combination of polymerases in conjunction with improved buffer and cycle conditions enables PCR amplification of long DNA fragments.

Tn*1546*-IR primer (table 2.4) is specific for both terminal inverted repeats of the transposon, and thus enables PCR amplification of the entire length of Tn*1546*-like elements. PCR was performed in 50µL reaction volumes containing 250ng genomic DNA, 30pmol Tn*1546*-IR primer, 350µM of each dNTP, 1.75mM $MgCl_2$ (Expand™ PCR buffer 1) and *Taq*/*Pwo* enzyme mix (0.75µL). Amplification was performed on a GeneAmp 9700 PCR system (PE Applied BioSystems, California) with the

protocol: (i) 94°C for 2 min; (ii) ten cycles of 94°C for 10s, 63°C for 30s and 68°C for 10 min; (iii) 25 cycles of 94°C for 10s, 63°C for 30s and 68°C for 10 min (elongation time increased 20s/cycle); and (iv) 68°C for 7 min.

**Table 2.4: Primers for the analysis of Tn*1546***

| Primer | Sequence | Coordinates | Reference |
|---|---|---|---|
| Tn*1546*-IR | 5′-GGAAAATGCGGATTTACAACGCTAAG | 13-38; 10839-10814 | Woodford *et al.* (1997a) |
| *orf1*-f | 5′-GATGGTGGCTCCTTTTCC | 906-923 | |
| *orf1*-r | 5′-CCATTGTTGGCAGCAGAC | 1597-1580 | |
| *vanR*-f | 5′-GCCGAAAAGCCCTTTGATA | 3841-3859 | |
| *vanR*-r | 5′-TCGTTCTAGTTTGGAATAGTCG | 4699-4678 | |
| *vanS*-f | 5′-TAGGGTAGAGCTTCCAGCGA | 5752-5771 | |
| *vanH*-r | 5′-ATTATCGTTGCCATAACGCC | 6115-6096 | |
| *vanX*-f | 5′-GATGAACGCTCTCATCATGC | 8448-8467 | |
| *vanY*-r | 5′-TTCCTGAGAAAACAGTGCTTCA | 9138-9117 | |
| *vanX*1 | 5′-ACTTGGGATAATTTCACCGG | 8082-8101 | Jensen *et al.* (1998) |
| *vanX*2 | 5′-TGCGATTTTGCGCTTCATTG | 8505-8486 | Jensen *et al.* (1998) |
| **Additional primers** | | | |
| 1216V-A | 5′-TTGAAGATGTAAGGCAGAGC | 8727-8746 | |
| 1216V-B | 5′-AGGCTGAACTGCCTGTTGAC | 8857-8838 | |
| 1216V-C | 5′-GATCGCATAGAGGGGTGGTA | 8802-8821 | |
| 1216V-D | 5′-GTACAGACCGAAAACCCGAA | 88-107[b] | |
| 1216V-E | 5′-GCAATTTCAGCAGGATGTGA | 714-695[b] | |
| 1542-A | 5′-TAGCTGTTCGAGCGAGTTCA | 980-961[a] | |
| 1542-B | 5′-TCGGCAATTTCATGTTCATC | 4022-4003 | |

Coordinates are based on the published Tn*1546* sequence; Genbank accession number M97297. Where no reference is listed the primer was designed by Primer3. Primers 1216V-A, -B and -C were used in the sequencing of the IS*1216V*-like element, whilst 1542-A and -B were used in the sequencing of IS*1542*. 1216V-D and -E were used to generate an IS1216V-like probe. [a] Coordinates correspond to the published sequence of IS*1542* (Genbank accession number AF114715; Darini *et al.*, 1999). [b] Co-ordinates correspond to the published sequence of the IS*1216V*-like element (Genbank accession number AF093508, Jensen, 1998).

### 2.3.14.ii Amplification of sequences within Tn*1546*

Primer pairs internal to the inverted repeats of Tn*1546* enabled amplification of those regions recognized as 'hot-spots' for variation, or those regions previously identified as harbouring variation by L-PCR and restriction analysis. PCR was performed according to the protocol described in section 2.3.3, with the exception that 200-500ng genomic DNA, prepared by guanidium thiocyanate extraction, was used as template. Primer annealing temperatures were adjusted accordingly. Any primers that failed to yield the anticipated product were reapplied with the Expand™ Long Template PCR System described above. In such cases, the elongation time was altered according to the manufacturer's guidelines.

The point mutation at nucleotide position 8234 (G→T) within the *vanX* gene, previously described by Jensen *et al.* (1998), was screened for by amplification of a 424-bp fragment internal to *vanX* (primers *vanX*1 and *vanX*2) and subsequent *Dde*I restriction of the amplicon (Palepou *et al.*, 1998).

### 2.3.15 Restriction analysis PCR amplicons

### 2.3.15.i Restriction of L-PCR amplicons

To enable comparisons of Tn*1546*-like elements, 0.5μL aliquots of the Tn*1546* L-PCR amplicons were digested with 10 U of *Cla*I or 10 U of *BamH*I restriction endonucleases (Promega). Restrictions were performed in 20μL volumes for 4 hours according to the manufacturer's instructions. Electrophoresis was performed alongside a GeneRuler™ DNA Ladder Mix (Helena BioSciences Ltd.) and the restriction fragments were subsequently sized with the BioRad Diversity Database, Gel Doc software.

### 2.3.15.ii Restriction of 'conventional' PCR amplicons

Those PCR amplicons obtained with the PCR protocol described in section 2.3.3 were restricted in 20μL volumes containing 10μL of PCR amplicon and 10 U of restriction endonuclease according to manufacturer's instructions. The restriction enzyme used was dependent on the anticipated sequence of the amplicon, based on the published sequence of Tn*1546* (GenBank accession number M97297). Suitable enzymes were determined with Webcutter v.2.0 (www.firstmarket.com/cutter/cut2.html).

### 2.3.16 Automated sequencing of PCR amplicons

PCR products of interest were sequenced to confirm the nature of the variation present within Tn*1546*-like elements. Fluorescent automated DNA sequencing with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and the ABI Prism 377 DNA Sequencer (Applied Biosystems, California, USA) was performed by DNASHEF Technologies (c/o Department of Haematology, Royal Infirmary of Edinburgh). Analysis of sequence data was performed with ABIView v.1.0 or Chromas v.1.56 software. The identity of the sequences obtained was established by electronic submission to the European Bioinformatics Institute (http://www2.ebi.ac.uk/fasta3/).

### 2.3.17 Transfer of DNA from agarose gel to nylon membrane

For several applications it was desirable to transfer DNA from agarose gels to nylon membranes to enable hybridization studies. This allowed probing of plasmid extractions and PFGE gels for the location of the *vanA* gene cluster, and

confirmation of the identity of DNA fragments derived from the restriction analysis of Tn*1546* L-PCR amplicons.

Alkali blotting by downward capillary action was employed. Alkali blotting has the advantage over Southern blotting that DNA is fixed to positively-charged nylon membranes during the transfer process. Downward capillary action was considered more efficient than upward capillary action in the transfer of large DNA fragments for which overnight transfer was necessary. In the course of upward capillary action the gel can become crushed, thus reducing the capillary action.

Following electrophoresis of DNA and visualization by ethidium bromide staining and UV illumination, gels were prepared for DNA transfer by depurination in 0.25M HCl (30 minutes) and denaturation in 0.4M NaOH (30 minutes). Downward capillary transfer of DNA to a positively-charged nylon membrane (Hybond™-$N^+$, Amersham Life Sciences Ltd., UK) was then set up according to the protocol described by Brown (1993) with 0.4M NaOH as transfer buffer. Transfer was allowed to continue for approximately 18 hours, after which the membrane was rinsed in 5x SSC (20 x SSC consists of 0.3M sodium citrate, 3M sodium chloride), wrapped in Saran wrap, and stored at –20°C until use.

### 2.3.18 Hybridization studies

Membrane-bound DNA prepared as above was probed with non-isotopically labelled probes and detected by chemiluminescence. Probes were either derived from PCR products or from oligonucleotide primers.

### 2.3.18.i Hybridization studies with probes derived from PCR products

PCR-derived probes were generated with the ECL™ Random Prime Labelling and Detection System (Amersham Life Sciences Ltd.). Nonamers of random sequence prime DNA synthesis on a denatured DNA template (in this case, denatured PCR product) in a reaction catalysed by exonuclease-free Klenow. Fluorescein-11-dUTP partially replaces dTTP in the labelling reaction, thus producing a fluorescein labelled probe. Anti-fluorescein-HRP (horseradish peroxidase) conjugate is applied to the membrane following hybridization, enabling bound probe to be detected by the presence of bound peroxidase with ECL detection reagents.

Probe labelling was performed according to the manufacturer's instructions, with PCR product (purified with the QIAquick PCR Purification kit; Qiagen, Crawley, UK) as template. Hybridization was performed overnight at 60°C in a Techne Hybridiser HB-1D with 20mL hybridization buffer, prepared according to the manufacturer's instructions. Probe concentrations ranged from 5ng/mL to 15ng/mL depending on the application. Stringency washes, blocking, antibody incubation and signal generation and detection were performed according to the manufacturer's instructions. The signal generated is in the form of blue light that was detected on Hyperfilm™-ECL (Amersham Life Sciences Ltd.). Exposure was performed for 3 hours at 4°C prior to developing the film in an Optimax processor (Jet X-Ray, London).

### 2.3.18.ii Hybridization studies with oligonucleotide probes

Fluorescein-labelled oligonucleotides were prepared by the ECL™ 3'-Oligolabelling and Detection System (Amersham Life Sciences Ltd.). The technique follows the same principle as that described above (section 2.3.18.i). The oligonucleotide is fluorescein-labelled through the action of terminal deoxynucleotidyl transferase that introduces a 3' tail of fluorescein-11-dUTP to the oligonucleotide.

Hybridization was performed overnight at 42°C in a Techne Hybridiser HB-1D. Oligonucleotide probe was applied at a concentration of 7.5-10ng/mL in 20mL-hybridization buffer. Stringency washes, blocking, antibody incubation and signal generation and detection were performed according to the manufacturer's instructions. Exposure of Hyperfilm™-ECL and developing of film was performed as above.

### 2.3.19 Growth curve analysis of GRE following glycopeptide challenge

In order to assess the response of different VanA GRE to glycopeptide challenge, log phase cultures were prepared and viable counts were performed at regular intervals. Cultures were challenged with vancomycin or teicoplanin after one hour, and viable counts continued at regular intervals for a further 6-7 hours. Colonies present on the viable count plates after overnight incubation were counted with an Anderman Colony Counter (Anderman, Kingston-upon-Thames, UK). $Log_{10}$[CFU/mL] was plotted against time to enable easy comparison of results.

### 2.3.20 Whole cell protein extraction

Cytoplasmic and membrane-associated protein was extracted from bacterial cells by a modified version of the protocol described by Arthur *et al.* (1996a). BHI broth (100mL) with or without 8mg/L vancomycin was inoculated with 8mL of overnight culture and incubated until the optical density at 600nm reached 0.7. Optical density was determined on a Bio-Rad SmartSpec™ 3000. Once cultures had reached the desired optical density, bacteria were harvested by centrifugation in a Sorvall RC5B centrifuge (5000 x *g* for 15 minutes at 4°C). Cells were then washed in 0.1M phosphate buffer (pH 7.0) and resuspended in 2.8mL of phosphate buffer supplemented with 2mg/mL lysozyme. Following incubation at 37°C for one hour, cells were lysed by sonication in an MSE Soniprep 150 (MSE Instruments, Crawley, UK). The lysate was centrifuged at 1000 x g for 10 minutes (to remove cell debris) prior to ultracentrifugation at 100,000 x g for one hour at 4°C in a Sorvall OTD 65B Ultracentrifuge. The supernatant (S100) was collected and the pellet (C100) resuspended in 2mL of 0.1M phosphate buffer containing 1% Triton X-100.

Protein concentration was estimated by the Bio-Rad Protein Assay on the SmartSpec™ 3000, with bovine serum albumen as a protein standard.

### 2.3.21 Enzyme assay for VanX dipeptidase activity

As an indication of glycopeptide resistance gene expression, the dipeptidase activity of VanX was estimated (with and without induction by vancomycin challenge) by determining the amount of D-Ala released from D-Ala-D-Ala with D-amino acid oxidase coupled to peroxidase as indicator reactions. The protocol was as described by Arthur *et al.* (1996a). Briefly, reagent A was freshly prepared by

mixing: 632μL of sterile deionized water, 1029μL of Tris-HCl (50mM), $MgCl_2$ (4mM), pH 7.0; 2880μL of disodium pyrophosphate (100mM, pH 8.3); 686μL of flavin adenine dinucleotide sodium salt (0.2mg/mL); 343μL of peroxidase diluted 1000-fold in water (Boehringer Mannheim); 34μL of D-amino acid oxidase (Boehringer Mannheim); 171μL of orthodianisidine (5mg/mL) and 225μL of 200mM D-Ala-D-Ala. Reagent A was preheated to 37°C and 525μL was added to 75μL aliquots of S100 extracts, diluted in 0.1M phosphate buffer as required. The $A_{460}$ was recorded on a Bio-Rad SmartSpec™ 3000 for one hour, taking readings every 150 seconds (2.5 minutes).

D,D-dipeptidase specific activity was defined as the number of nmoles of D-Ala formed per minute per mg of protein present in the extract. To calculate this, the change in absorbance associated with the complete hydrolysis of known concentrations of D-Ala was established. The change in absorbance seen in the VanX enzyme assays could thus be correlated to known concentrations of D-Ala, thus enabling calculation of the number of moles of D-Ala being formed.

### 2.3.22 Enzyme assay for VanY carboxypeptidase activity

VanY D,D-carboxypeptidase activity in C100 extracts was determined as for the VanX enzyme assays with the following modifications. The pentapeptide L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala (Boehringer Mannheim) replaced D-Ala-D-Ala as substrate, at a final concentration of 0.21mM. Dilutions of C100 extracts were performed in 0.1M phosphate buffer (pH 7.0) containing 1% Triton X-100, and $ZnSO_4$ was added at a final concentration of 5mM.

# Chapter 3: Results

## Species identification, *van* genotyping & PFGE analysis

For the purposes of this chapter, the enterococcal isolates studied will be assigned to two distinct groups: 'Nosocomial GRE isolates' and 'Enterococcal epidemiology study isolates'. 'Nosocomial GRE isolates' refers to those isolates collected from Scottish hospitals in the course of the five-year study period. These isolates will be discussed separately from those enterococcal isolates collected during the five-month study of enterococcal epidemiology.

## 3.1 Characterization of the nosocomial GRE isolates

### 3.1.1 Species identification and *van* genotyping of nosocomial GRE

Over the five-year period from January 1995 to December 1999, 55 isolates of GRE were collected from clinical diagnostic laboratories across Scotland, representing eight different hospitals. As summarized in table 3.1, the majority of GRE isolated were from the Royal Infirmary of Edinburgh (RIE), with many of these being isolated during the course of an outbreak of GRE infection in 1995. The outbreak, which occurred in the hospital's renal unit, ran from January to September of that year, during which time 30 GRE were isolated. Whilst four of these isolates were unavailable for further study, the remaining 26 isolates are amongst the 55 isolates summarized in table 3.1. The remainder of the 55 isolates consist primarily of sporadic isolates from the RIE and the other hospitals listed. Relevant details for all 55 GRE isolates are listed in tables 3.4 and 3.5 (pages 92 and 93).

All GRE were identified to the species level by the API20 Strep system, and all were found to be either *E. faecium* (64%) or *E. faecalis* (36%).

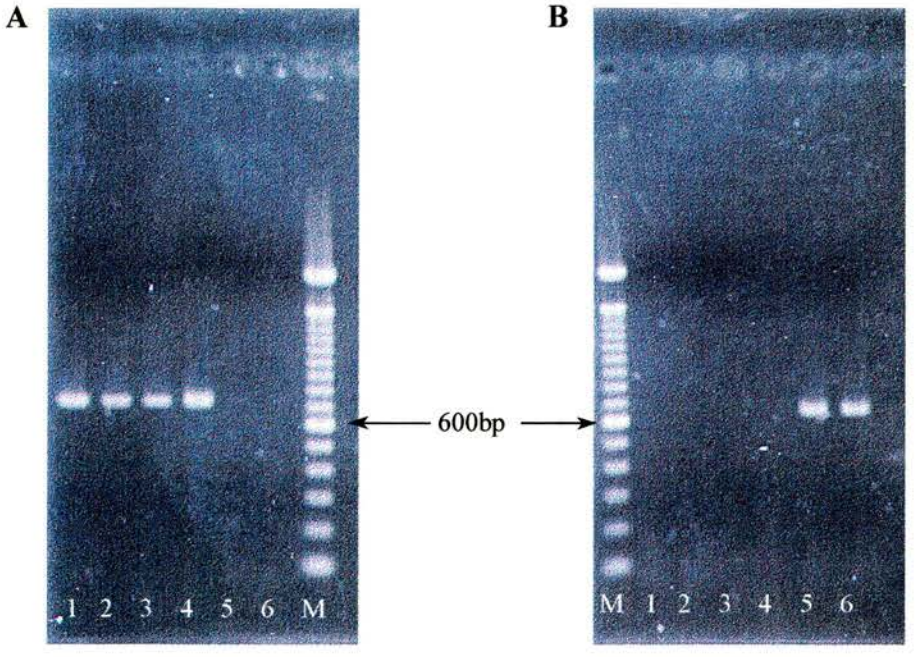
The clinical laboratories had identified all isolates as vancomycin-resistant at the time of their isolation. This was subsequently confirmed by vancomycin and

teicoplanin susceptibility testing, which also enabled identification of the glycopeptide resistance phenotype. All 55 isolates had glycopeptide resistance characteristic of either the VanA or VanB phenotype. This was confirmed by PCR detection of the *vanA* and *vanB* resistance genes. Whilst the use of the *vanA* and *vanB*-specific primers in multiplex PCR has been described (Dutka-Malen *et al.*, 1995), initial attempts at multiplex PCR failed. Each isolate was therefore tested for the *vanA* and *vanB* genes, with the primers in two separate PCR reactions. Figure 3.1 shows the PCR amplicons obtained with the *vanA* and *vanB*-specific primers.

**Table 3.1: Source and glycopeptide resistance genotypes of nosocomial GRE isolates**

| HOSPITAL | *vanA*$^+$ | *vanB*$^+$ | *vanA/B*$^+$ | Total |
|---|---|---|---|---|
| Royal Infirmary of Edinburgh | 22 | 5 | 4 | **31** |
| Western General Hospital, Edinburgh | 1 | - | - | **1** |
| Edinburgh City Hospital | 2 | - | - | **2** |
| Borders General Hospital, Melrose | 2 | - | - | **2** |
| Monklands Hospital, Airdrie | 2 | - | - | **2** |
| Ninewells Hospital, Dundee | 4 | - | - | **4** |
| Western Infirmary, Glasgow | 9 | 1 | 1 | **11** |
| Aberdeen Royal Infirmary | 1 | 1 | - | **2** |
| **TOTAL** | **43** | **7** | **5** | **55** |

## Figure 3.1: *vanA* and *vanB*-specific PCR




PCR with *vanA*-specific primers (A) and *vanB*-specific primers (B). Both gels depict PCR reactions performed on known VanA isolates (lanes 1-4) and VanB isolates (lanes 5 & 6). The *vanA* amplicon is 732-bp, whilst the *vanB* amplicon is 635-bp. The molecular marker (M) is a 100-bp DNA ladder.

The majority of isolates were positive for either *vanA* or *vanB*, although five isolates were positive for both resistance genes. These 'double-positive' isolates generally displayed the VanA phenotype, although teicoplanin susceptibility varied between MIC determinations. For the remainder of the thesis, these five isolates will be referred to as VanA, thus bringing the total number of VanA isolates to 48.

Table 3.1 shows the breakdown of resistance phenotypes within the various hospitals studied, whilst table 3.2 summarizes the levels of resistance detected in the isolates of different resistance phenotypes. When comparing the breakdown of glycopeptide resistance phenotypes presented in table 3.1, it should be noted that isolates collected from Ninewells Hospital, Dundee, and the Western Infirmary, Glasgow, were collected on the premise that they were VanA isolates. VanB isolates were not referred for study, and thus the numbers presented from these two hospitals do not accurately reflect the prevalence of VanA and VanB enterococci in the two hospitals. The one VanB isolate from Glasgow's Western Infirmary was received in error.

**Table 3.2: Ranges of glycopeptide susceptibilities displayed by VanA, VanB and 'double-positive' (*vanA/vanB*-positive) isolates**

| Resistance genotype | (n) | Vancomycin MIC range (mg/L) | Teicoplanin MIC range (mg/L) |
|---|---|---|---|
| *vanA*$^+$ | 43 | 64 - 1024 | 8 - 256 |
| *vanB*$^+$ | 7 | 16 - 1024 | ≤ 1 - 32[a] |
| *vanA/B*$^+$ | 5 | 64 - 512 | ≤ 1 - 64[b] |

[a] One VanB isolate had a teicoplanin MIC of 32mg/L. All other VanB isolates were teicoplanin sensitive (MIC ≤ 4mg/L). [b] One *vanA/B*$^+$ isolate displayed the VanB phenotype, being susceptible to teicoplanin.

All *vanA*-positive GRE were later studied for their ability to transfer glycopeptide resistance (chapter 4). To enable the identification of antibiotic resistance determinants that were co-transferred with the *van* genes, a range of antibiotic sensitivity tests were performed on all VanA isolates. The breakpoints used in the definition of resistance are shown in table 3.3.

**Table 3.3: Breakpoints adopted for the definition of antibiotic resistance**

| | mg/L | |
|---|---|---|
| **Antibiotic** | **Sensitive** | **Resistant** |
| Ampicillin | ≤ 2 | ≥ 16 |
| Tetracycline | ≤ 4 | ≥ 16 |
| Erythromycin | ≤ 0.5 | ≥ 8 |
| Chloramphenicol | ≤ 8 | ≥ 32 |
| Streptomycin | | ≥ 2000 [a] |
| Gentamicin | | ≥ 500 [a] |

[a] High-level resistance

Erythromycin resistance was exhibited by 88% of the VanA isolates, being common amongst both *E. faecium* and *E. faecalis*. Ampicillin resistance was encountered more commonly in *E. faecium* than in *E. faecalis* (75% versus 19%; $P < 0.001$). Conversely, tetracycline resistance was more common in *E. faecalis* than in *E. faecium* (94% versus 38%; $P < 0.001$). Fifty-eight percent of the VanA isolates displayed high-level streptomycin resistance (HLSR), with 10% displaying high-level gentamicin resistance (HLGR). All but one of the isolates that displayed HLGR also displayed high-level resistance to streptomycin. Chloramphenicol resistance was rarely encountered in the VanA isolates studied.

Table 3.4 summarizes the site of isolation of the 55 GRE isolates studied. The preponderance of faecal isolates reflects the routine faecal screening implemented in April 1995 by the renal unit of the Royal Infirmary of Edinburgh in response to the GRE outbreak. Twenty of the 26 isolates originating from the renal unit outbreak were isolated from faecal samples. In addition, the majority of GRE isolates from Glasgow's Western Infirmary (82%) were faecal samples, being isolated during the course of a 30-month investigation examining the prevalence of GRE.

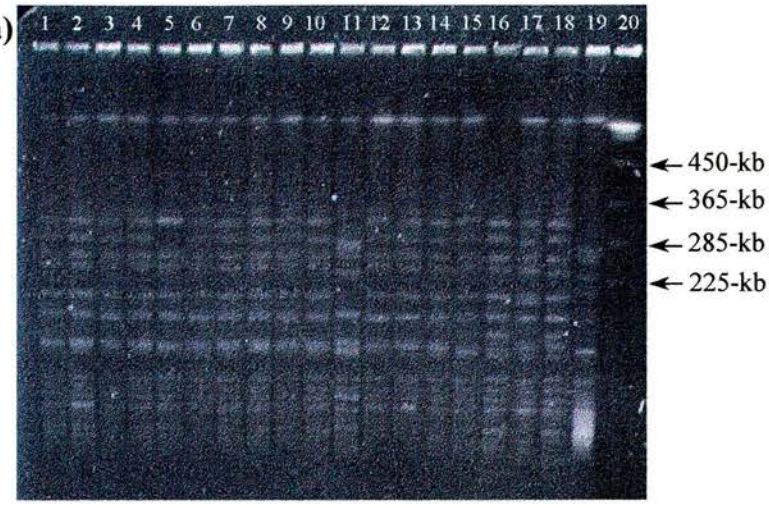
**Table 3.4: Sites of GRE isolation**

| Sites of isolation | Number (%) |
|---|---|
| Faecal | 32 (58) |
| Urine | 9 (16) |
| Wound Infection | 1 (2) |
| Blood | 1 (2) |
| Other | 6 (11) |
| Unknown | 6 (11) |

### 3.1.2 PFGE analysis of nosocomial GRE isolates

PFGE analysis was performed on all 55 GRE isolates. Figure 3.2 illustrates the main PFGE types recognized. Interpretation of the results was performed in two ways: (i) visual comparison of banding patterns and interpretation according to the criteria described by Tenover *et al.* (1995), and (ii) comparison with the Bio-Rad Diversity Database computer software.

## Figure 3.2: PFGE analysis of nosocomial GRE isolates

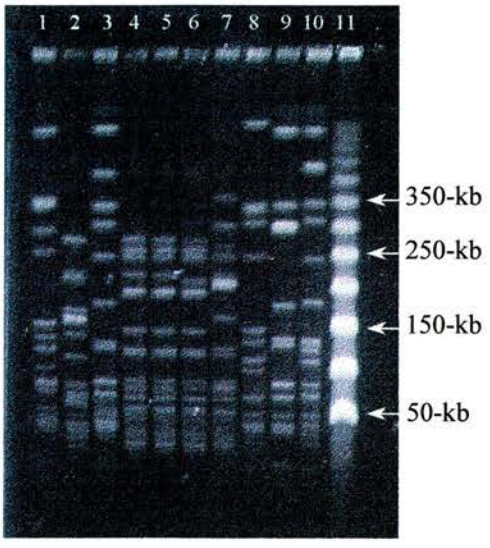



Lane: 1 - RIE-01; 2 - RIE-02; 3 - RIE-03; 4 - RIE-04; 5 - RIE-05; 6 - RIE-06; 7 - RIE-07; 8 - WGH-01; 9 - RIE-08; 10 - RIE-09; 11 - RIE-10; 12 - BGH-01; 13 - BGH-02; 14 - RIE-11; 15 - RIE-12; 16 - RIE-13; 17 - RIE-14; 18 - RIE-15; 19 - RIE-16; 20 - *Saccharomyces cerevisiae* DNA ladder.


Lane: 1 - RIE-18; 2 - RIE-19; 3 - ECH-01; 4 - RIE-23; 5 - RIE-24; 6 - RIE-25; 7 - ECH-02; 8 - RIE-26; 9 - RIE-27; 10 - RIE-29; 11 - MON-01; 12 - MON-02; 13 - RIE-30; 14 - ARI-01; 15 - NIN-01; 16 - NIN-02; 17 - NIN-03; 18 - NIN-04; 19 - GWI-02; 20 - 50-1000 kb DNA ladder.

**(Figure 3.2 cont.: PFGE analysis of nosocomial GRE isolates)**

**(c)**




Lane: 1 - GWI-03; 2 - GWI-04;
3 - GWI-05; 4 - GWI-06;
5 - GWI-07; 6 - GWI-08;
7 - GWI-09; 8 - GWI-10;
9 - GWI-11; 10 - RIE-31;
11 - 50-1000 kb DNA ladder.

### 3.1.2.i Visual comparison of PFGE patterns

Initial interpretation of PFGE patterns was performed by visual comparison and adoption of the criteria described by Tenover and colleagues (Tenover *et al.*, 1995). Tables 3.5 and 3.6 list the relevant details of all 55 GRE isolates, including the assigned PFGE types, as determined by the visual comparison of restriction patterns.

As outlined in section 3.1.1, 26 of the 55 nosocomial GRE isolates collected over the five-year period originated from an outbreak in the renal unit of the Royal Infirmary of Edinburgh in 1995. Visual comparison of the PFGE patterns obtained from these 26 isolates revealed that a significant proportion of the isolates shared a common PFGE pattern, and were thus designated the 'outbreak strain'. Nine VanA *E. faecium* isolates exhibited identical PFGE restriction patterns (PFGE type A: the outbreak strain), with a further seven isolates deemed to be closely related to this group, on the basis that they differed by no more than three bands. These seven isolates were therefore assigned to PFGE subtypes $A_1$-$A_7$ (table 3.5).

**Table 3.5: Relevant information on GRE isolates from the Royal Infirmary of Edinburgh**

| Isolate | Species | *van* gene | MIC (mg/L) | | | PFGE type |
|---|---|---|---|---|---|---|
| | | | VA | TE | AV | |
| **Renal unit isolates** | | | | | | |
| RIE-01 | FM | A | 512 | 16 | 512 | A |
| RIE-02 | FM | A | 1024 | 32 | >512 | A |
| RIE-03 | FM | A | 1024 | 64 | >512 | A |
| RIE-04 | FM | A | 1024 | 64 | >512 | A |
| RIE-05 | FM | A | 1024 | 64 | >512 | $A_1$ |
| RIE-06 | FM | A | 1024 | 64 | >512 | A |
| RIE-07 | FM | A | 1024 | 32 | >512 | A |
| RIE-08 | FM | A | 1024 | 64 | >512 | A |
| RIE-09 | FM | A | 1024 | 32 | >512 | A |
| RIE-10 | FM | A | 256 | 16 | >512 | $A_2$ |
| RIE-11 | FM | A | 1024 | 64 | 512 | A |
| RIE-12 | FM | A | 1024 | 32 | >512 | $A_3$ |
| RIE-13 | FM | A | 1024 | 32 | >512 | $A_4$ |
| RIE-14 | FM | A+B | 64 | <1 | 8 | $A_5$ |
| RIE-15 | FM | A | 1024 | 32 | >512 | $A_6$ |
| RIE-16 | FM | A | 1024 | 32 | 512 | |
| RIE-17 | FM | B | 128 | 0.5 | >512 | $A_7$ |
| RIE-18 | FM | A | 512 | 32 | >512 | B |
| RIE-19 | FM | A+B | 512 | 32 | 16 | B |
| RIE-21 | FS | B | 16 | 0.5 | 16 | $C_1$ |
| RIE-22 | FS | B | 64 | 0.5 | 16 | $C_2$ |
| RIE-24 | FS | A+B | 512 | 64 | 512 | C |
| RIE-25 | FS | A | 64 | 64 | >512 | $D_1$ |
| RIE-26 | FS | A+B | 64 | 16 | 256 | $D_2$ |
| RIE-27 | FS | A | 128 | 64 | >512 | |
| RIE-28 | FS | B | 1024 | 32 | 32 | |
| **Non-renal unit isolates** | | | | | | |
| RIE-20 | FS | B | 64 | 1 | 1 | $C_3$ |
| RIE-23 | FS | A | 1024 | 32 | 256 | C |
| RIE-29 | FS | A | 128 | 64 | >512 | $D_3$ |
| RIE-30 | FM | A | 1024 | 16 | ND | |
| RIE-31 | FS | A | 64 | 8 | ND | |

FM - *E. faecium*; FS - *E. faecalis*. MIC - Minimum Inhibitory Concentration. VA - Vancomycin; TE - Teicoplanin; AV - Avoparcin. ND - Not Determined. PFGE types were assigned on the basis of visual comparison and interpretation according to the criteria described by Tenover *et al.* (1995). Where no PFGE type is specified the isolate is unrelated to any other.

**Table 3.6: Relevant information on GRE isolated from hospitals other than the Royal Infirmary of Edinburgh**

| Isolate | Species | *van* gene | MIC (mg/L) | | | PFGE type |
|---|---|---|---|---|---|---|
| | | | VA | TE | AV | |
| **Western General Hospital, Edinburgh** | | | | | | |
| WGH-01 | FM | A | 1024 | 32 | >512 | A |
| **Borders General Hospital, Melrose** | | | | | | |
| BGH-01 | FM | A | 1024 | 32 | >512 | A |
| BGH-02 | FM | A | 1024 | 128 | >512 | A |
| **Edinburgh City Hospital** | | | | | | |
| ECH-01 | FM | A | 128 | 64 | 64 | |
| ECH-02 | FS | A | 256 | 256 | >512 | $D_4$ |
| **Monklands Hospital, Airdrie** | | | | | | |
| MON-01 | FM | A | 512 | 32 | ND | |
| MON-02 | FS | A | 256 | 128 | ND | $D_5$ |
| **Aberdeen Royal Infirmary** | | | | | | |
| ARI-01 | FM | A | 128 | 64 | ND | |
| ARI-02 | FM | B | 16 | 0.5 | ND | ND |
| **Ninewells Hospital, Dundee** | | | | | | |
| NIN-01 | FM | A | 128 | 64 | ND | |
| NIN-02 | FS | A | 64 | 16 | ND | E |
| NIN-03 | FS | A | 128 | 16 | ND | E |
| NIN-04 | FS | A | 64 | 8 | ND | |
| **Western Infirmary, Glasgow** | | | | | | |
| GWI-01 | FM | B | 64 | 0.5 | ND | |
| GWI-02 | FM | A | 128 | 64 | ND | |
| GWI-03 | FS | A | 64 | 8 | ND | |
| GWI-04 | FM | A | 512 | 64 | ND | |
| GWI-05 | FS | A | 128 | 8 | ND | |
| GWI-06 | FM | A | 1024 | 64 | ND | F |
| GWI-07 | FM | A | 512 | 16 | ND | F |
| GWI-08 | FM | A | 64 | 16 | ND | $F_1$ |
| GWI-09 | FM | A+B | 256 | 16 | ND | |
| GWI-10 | FS | A | 128 | 8 | ND | |
| GWI-11 | FS | A | 64 | 8 | ND | |

FM - *E. faecium*; FS - *E. faecalis*. MIC - Minimum Inhibitory Concentration. VA - Vancomycin; TE - Teicoplanin; AV - Avoparcin. ND - Not Determined. PFGE types were assigned on the basis of visual comparison and interpretation according to the criteria described by Tenover *et al.* (1995). Where no PFGE type is specified the isolate is unrelated to any other.

Interestingly, of the seven isolates assigned to PFGE subtypes $A_1$-$A_7$ (subtypes of the outbreak strain), one harboured both the *vanA* and *vanB* genes, whilst one harboured solely the *vanB* gene. However, the isolate harbouring both the *vanA* and *vanB* genes (RIE-14; PFGE subtype $A_5$) exhibited the VanB phenotype, suggesting that the *vanA* gene cluster may have been inactive.

The remaining isolates from the renal unit outbreak were deemed to be more heterogeneous in nature following visual comparison of PFGE patterns, although small discrete clusters of related isolates were recognized (Table 3.5). Two isolates of *E. faecium* (RIE-18 and RIE-19) shared identical PFGE patterns and were designated PFGE type B. A further two clusters of related isolates were recognized within the renal unit isolates, being classed as PFGE types C and D. Three isolates (RIE-21, RIE-22 and RIE-24) were deemed to be closely related to one another, and were assigned to PFGE type C and subtypes $C_1$ and $C_2$. Two further isolates (RIE-25 and RIE-26) were also deemed to be closely related to one another and were assigned to PFGE types $D_1$ and $D_2$.

PFGE analysis of isolates from outwith the renal unit outbreak revealed a predominantly polyclonal collection of GRE. However, some non-renal unit isolates were found to be related to isolates originating from the renal unit outbreak. Most notably, three VanA *E. faecium* isolates, two from the Borders General Hospital, Melrose, and one from Edinburgh's Western General Hospital, displayed identical PFGE patterns to the outbreak strain (PFGE type A) identified within the renal unit of the Royal Infirmary of Edinburgh (table 3.6). A further five non-renal unit isolates showed varying degrees of similarity to renal unit isolates by PFGE analysis. Isolate RIE-23 from the medical ward of the Royal Infirmary of Edinburgh had an

identical PFGE pattern to RIE-24 from the renal unit (PFGE type C), whilst RIE-20 from the transplant ward was closely related (table 3.5). Finally, three non-renal unit isolates were deemed to be 'possibly related' to RIE-25 and RIE-26 from the renal unit. These three isolates (designated PFGE types $D_3$-$D_5$) included one isolate from Edinburgh City Hospital and one from Monkland's Hospital, Airdrie (table 3.6).

Two VanA *E. faecalis* isolates from Dundee's Ninewells Hospital shared an identical PFGE pattern (type H), whilst three VanA *E. faecium* isolates from Glasgow's Western Infirmary were closely related to one another (PFGE type J and subtype $J_1$). PFGE types H and J were distinct from each other, and from those PFGE types discussed above. All other GRE isolates studied by PFGE had unique PFGE patterns and were deemed to be unrelated to any other isolates.

### 3.1.2.ii Computer-aided comparison of PFGE patterns

Latterly it was decided to re-evaluate the similarity between PFGE patterns with the Bio-Rad Diversity Database software. The criteria defined by Tenover *et al.* are "intended to be used ... to examine relatively small sets of isolates (typically $\leq 30$) related to putative outbreaks of disease" (Tenover *et al.*, 1995). Whilst these criteria could thus be applied to the group of isolates comprising the renal unit outbreak in 1995, they were not ideally suited to the study of the complete set of isolates. The issue of PFGE interpretation will be addressed in the discussion (chapter 7).

The gel images introduced into the Diversity Database were taken from the original Polaroid photographs. This, however, had a disadvantage, as the gels were not initially run with a view to the computer-aided comparison being made. Ideally, the gels should be run with at least two DNA ladders flanking the samples, and preferably one further DNA ladder situated in the middle of the gel.

Diversity Database analysis was performed on the three gel photographs depicted in figure 3.2. Samples do not electrophorese uniformly across the width of a gel, and thus a sample in the first lane of a gel will seldom be directly comparable to a DNA ladder in the last lane of a gel. It was therefore anticipated that isolates deemed to be related by visual comparison of PFGE patterns would fail to be recognized as related by the computer software, which is dependent on the calculated size of the DNA fragments being compared.

This did indeed prove to be the case, and was particularly evident in the analysis of the outbreak strain (PFGE type A) depicted in figure 3.2a. Rather than the one outbreak strain (as had been recognized by visual comparison of the PFGE patterns), the phylogenetic tree obtained through Diversity Database analysis suggested several small clusters of related isolates. The nine isolates that were judged to be identical by eye, ranged in similarity from 56% to 96% when analysed by the Diversity Database, and the seven isolates deemed to be closely related were shown to have 57% to 86% similarity with the PFGE type A strain. The phylogenetic tree obtained through analysis of figure 3.2a is shown in appendix A.

Similar findings were obtained with analysis of those gels depicted in figure 3.2b and 3.2c. Whilst the Diversity Database identified RIE-23 and RIE-24 as being 'identical' (89% similarity), it failed to detect identity between isolates RIE-18 and RIE-19 (63% similarity). GWI-06 and GWI-07, previously identified as having identical PFGE patterns, were calculated as 88% similar, with the closely related isolate GWI-08 showing 77% similarity (see appendices B and C).

All isolates deemed to be unrelated to any other isolates by visual comparison of PFGE patterns were confirmed as such by Diversity Database analysis, with

similarity typically under 50%. The only exception to this was with isolates GWI-05 and RIE-31 (figure 3.2c), previously considered unrelated. Diversity Database analysis calculated the two isolates to have 65% similarity. Whilst such percentage similarity would ordinarily indicate that the strains were unrelated, it was thought this may be significant, given the variation seen in percentage similarity of 'identical' strains.

## 3.2 Characterizaton of isolates from five-month epidemiology survey

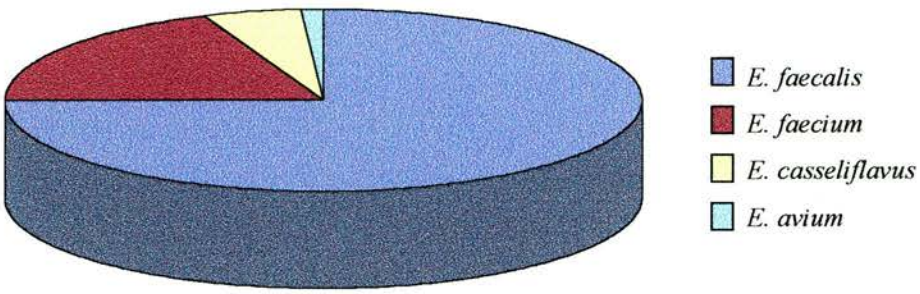
### 3.2.1 Species identification and *van* genotyping of isolates

During the course of the five-month survey of enterococcal epidemiology, 94 isolates were collected from the diagnostic laboratories of the Royal Infirmary of Edinburgh. Of these 94 isolates, 49 were hospital isolates and 26 general practice isolates. The remaining 19 isolates were obtained through the routine screening of faecal samples.

Figure 3.3 demonstrates the proportion of isolates belonging to different species, as determined with the API20 Strep system. As anticipated, *E. faecalis* was the predominant species, with *E. faecium* accounting for the majority of the remainder.

The API20 Strep system identified five isolates as *E. casseliflavus* and one as *E. avium*; all six isolates being obtained from the routine screening of faecal samples. Attempts were made to amplify the *E. casseliflavus* species-specific *vanC*2/3 gene from those five isolates identified as *E. casseliflavus*, with the primers described by Dutka-Malen *et al.* (1995). PCR analysis failed to demonstrate the presence of the *vanC*2/3 gene, thus casting doubt over the API identification.

**Figure 3.3: Proportion of enterococcal isolates belonging to different species**




According to API20 Strep system identification

All isolates were screened for vancomycin resistance with a 5μg vancomycin disc. Of the 94 isolates, two were identified as being glycopeptide-resistant. Glycopeptide susceptibility testing suggested both isolates, one *E. faecalis* and one *E. faecium*, were VanA phenotype, and this was subsequently confirmed by PCR detection of the *vanA* gene. Both isolates were general practice isolates from different individuals residing in the Penicuik area, situated to the south of Edinburgh.

### 3.2.2 PFGE analysis of enterococcal isolates

All 94 isolates collected during the five-month period were analysed by PFGE and the resulting patterns interpreted according to the criteria described by Tenover *et al.* (1995), as outlined previously.

Within the 94 isolates, ten clusters of related isolates were identified, designated PFGE types I-X so as to avoid confusion with those PFGE types recognized previously. The largest cluster (PFGE type I) consisted of nine *E. faecium* isolates, whilst the remaining clusters consisted of *E. faecalis* isolates. Table 3.7 summarizes the ten clusters of related isolates.

**Table 3.7: Summary of PFGE types I-X**

| PFGE type | Number of isolates [a] | Source of isolates |
|---|---|---|
| I | 9 | RIE, ECH, General practice |
| II | 7 | RIE |
| III | 4 | RIE, General practice |
| IV | 4 | General practice |
| V | 3 | RIE |
| VI | 2 | RIE, General practice |
| VII | 2 | ECH, General practice |
| VIII | 2 | RIE, General practice |
| IX | 2 | SMMP, General practice |
| X | 2 | SLTU, RIE |

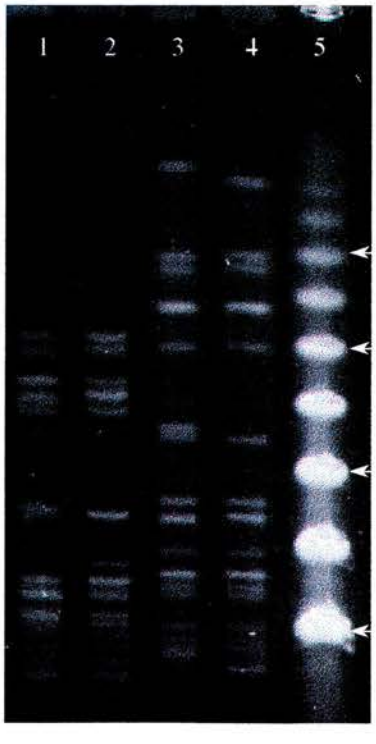
RIE - Royal Infirmary of Edinburgh; ECH - Edinburgh City Hospital; SMMP - Simpson's Memorial Maternity Hospital; SLTU - Scottish Liver Transplant Unit. [a] Number of isolates of each PFGE type, including sub-types.

Whilst PFGE types II, V and X consisted solely of nosocomial isolates, and PFGE type IV consisted solely of general practice isolates, many of the PFGE types included both nosocomial and general practice isolates, suggesting strains that were common to both the community and hospital settings. All the isolates represented in table 3.7 are glycopeptide-sensitive. The two VanA general practice isolates identified in the course of the study were unique by PFGE, as were all remaining isolates. Amongst the isolates represented in table 3.7 are two of the isolates

identified as *E. casseliflavus* by API20 Strep identification. One such isolate was of PFGE type I and thus appeared to be a misidentified *E. faecium*, whilst the other was of PFGE type VII and was therefore thought to be an *E. faecalis* isolate.

The PFGE patterns exhibited by the 94 isolates were compared to the PFGE patterns displayed by the nosocomial GRE described previously. The two VanA GRE from general practice specimens were unrelated to nosocomial GRE. However, PFGE types I and II appeared related to the previously identified PFGE types B and D that were described in section 3.1.2. Representative isolates were re-examined by PFGE so they could be viewed together on the same gel (figure 3.4).

**Figure 3.4: PFGE analysis of glycopeptide-sensitive and -resistant enterococci**




Lanes: 1 - PFGE type B (RIE-18); 2 - PFGE type I; 3 - PFGE type $D_3$ (RIE-29); 4 - PFGE type II; 5 - 50-1000 kb DNA ladder

As shown in figure 3.4, PFGE types I and II, displayed by glycopeptide-sensitive enterococci, were closely related to PFGE types B and D, respectively, that were

displayed by glycopeptide-resistant enterococci. The glycopeptide-sensitive enterococci belonging to PFGE types I and II appeared to be widespread within the Royal Infirmary of Edinburgh, being isolated from many different locations within the hospital. Amongst the wards from which PFGE types I and II were isolated were the renal unit and GI/Liver wards. The same wards harboured GRE isolates of PFGE types B and D, these isolates being identified two to three years earlier than the glycopeptide-sensitive organisms.

# Chapter 4: Results

## Transferability and location of Tn*1546*

## 4.1 Transferability of Tn*1546*

The 48 nosocomial GRE isolates identified as harbouring Tn*1546* were used as donor cells in filter mating experiments with a suitable recipient cell. All *E. faecium* isolates were mated with *E. faecium* GE-1, whilst all *E. faecalis* isolates were mated with *E. faecalis* JH2-2. Both recipients are glycopeptide-sensitive, but resistant to rifampicin and fusidic acid, thus allowing selection of glycopeptide-resistant transconjugants on suitable selective media.

Thirty-eight of the 48 *vanA*-positive GRE isolates (81%) successfully transferred glycopeptide resistance. All transconjugants displayed VanA phenotype resistance, including the transconjugant derived from RIE-14 (*vanA/B*$^{+}$; VanB phenotype). One transconjugant colony was picked from each successful mating, sub-cultured and stored at -70°C to enable further study. One transconjugant (arising from donor GWI-06) was lost during storage. The remaining 37 transconjugants were screened for co-transfer of resistance determinants. Erythromycin, chloramphenicol, ampicillin, tetracycline, streptomycin and gentamicin susceptibility tests were performed on all donors and transconjugants. The results of these susceptibility tests are summarized in table 4.1.

The two recipients used in the conjugation experiments were found to be sensitive to all agents tested, with the exception of *E. faecium* GE-1, which displayed tetracycline resistance (MIC 32mg/L). This prevented the identification of tetracycline resistance gene transfer from *E. faecium* isolates. Ten of the 37 transconjugants (27%) had acquired erythromycin resistance in addition to glycopeptide resistance, whilst chloramphenicol resistance, HLSR and HLGR were only rarely co-transferred. No transfer of ampicillin resistance was detected.

**Table 4.1: Details of VanA donor strains and resulting transconjugants**

| Transconjugant | Donor | Species | Resistance profile of donor [a] | Co-transferred resistance determinants |
|---|---|---|---|---|
| ARB-01 | RIE-01 | FM | Em, Amp, HLSR | |
| ARB-02 | RIE-02 | FM | HLSR | |
| ARB-03 | RIE-03 | FM | Em, Amp, Tet, HLSR | |
| ARB-04 | RIE-04 | FM | Em, Amp, Tet, HLSR | |
| ARB-05 | RIE-05 | FM | Em, Amp, HLSR | |
| ARB-06 | RIE-06 | FM | Em, Amp, HLSR | |
| ARB-07 | RIE-07 | FM | Em, Amp, HLSR | |
| ARB-08 | WGH-01 | FM | Amp, HLSR | |
| ARB-09 | RIE-08 | FM | Em, Amp, Tet, HLSR | |
| ARB-10 | RIE-09 | FM | Em, Amp, Tet, HLSR | |
| ARB-11 | RIE-10 | FM | Em, Amp, Tet, HLSR | |
| ARB-12 | BGH-01 | FM | Em, Amp, HLSR | |
| ARB-13 | BGH-02 | FM | Em, Amp, HLSR | |
| ARB-14 | RIE-11 | FM | Em, Amp, HLSR | |
| ARB-15 | RIE-12 | FM | Em, Amp | |
| ARB-16 | RIE-13 | FM | Em, Amp, Tet, HLSR | |
| ARB-17 | RIE-14 | FM | Em, Amp, Tet, HLSR | |
| ARB-18 | RIE-15 | FM | Em, Amp, Tet, HLSR | |
| ARB-19 | RIE-16 | FM | Em, Amp, HLSR | |
| ARB-20 | RIE-18 | FM | Em, Amp, HLSR | Em |
| ARB-21 | RIE-19 | FM | Em, HLSR | Em, HLSR |
| ARB-22 | ECH-01 | FM | - | |
| ARB-23 | RIE-23 | FS | Em, Amp, Tet, HLSR | Tet |
| ARB-24 | RIE-24 | FS | Em, Amp, HLSR | Tet |
| ARB-25 | ECH-02 | FS | Em, Tet, HLSR | Em, HLSR |
| ARB-26 | RIE-27 | FS | Em, Amp, Tet, HLSR | Tet |
| ARB-27 | RIE-29 | FS | Em, Tet, HLSR, HLGR | Tet, HLGR |
| ARB-28 | MON-01 | FM | Amp, Tet | |
| ARB-29 | ARI-01 | FM | Em, Tet | |
| ARB-30 | RIE-30 | FM | Em, Amp, Tet, HLSR | |
| ARB-31 | NIN-03 | FS | Em, Tet | Em |
| ARB-32 | NIN-04 | FS | Em, Tet | Em |
| ARB-33 | GWI-07 | FM | Em, Amp | Em |
| ARB-34 | GWI-08 | FM | Em | Em |
| ARB-35 | GWI-10 | FS | Em, Tet | Em |
| ARB-36 | GWI-11 | FS | Em, Tet | Em |
| ARB-37 | RIE-31 | FS | Em, Cm, Tet, HLSR | Em, Cm, Tet, HLSR |

FM – *E. faecium*; FS – *E. faecalis*. [a] Resistance profile following erythromycin (Em), ampicillin (Amp), chloramphenicol (Cm), tetracycline (Tet), streptomycin and gentamicin susceptibility testing. HLSR & HLGR – High-level resistance to streptomycin and gentamicin respectively. Co-transferred resistance determinants are in addition to glycopeptide resistance. The co-transfer of tetracycline resistance from *E. faecium* isolates could not be detected due to the tetracycline resistance in *E. faecium* GE-1.

The co-transfer of tetracycline resistance from *E. faecalis* isolates was detected, and it is thought likely that similar transfer from *E. faecium* isolates would have been witnessed, had a suitable recipient been used. Alternatively, the presence of tetracycline resistance determinants (e.g. *tetM*) within *E. faecium* transconjugants could have been detected by probing. It was noticeable that many *E. faecium* transconjugants had a tetracycline MIC 4- to 8-fold higher than that of the recipient. This may reflect the transfer of tetracycline resistance from the donor.

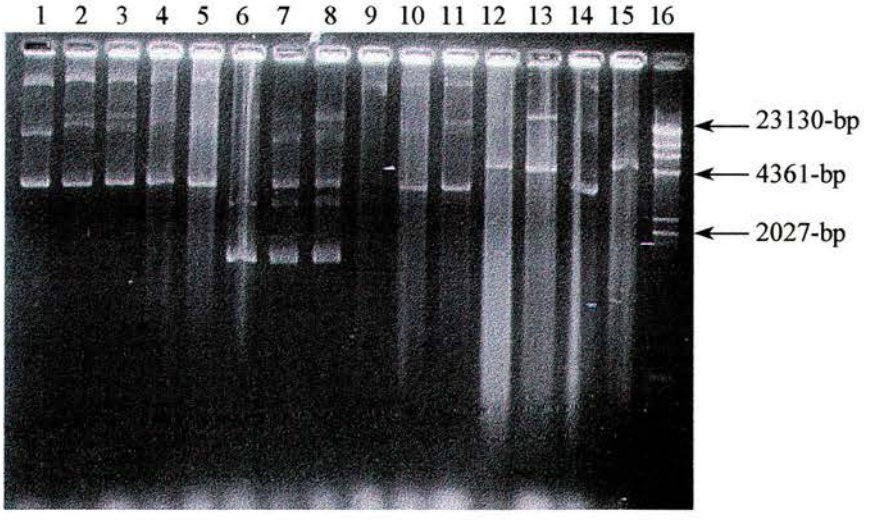
## 4.2 Analysis of plasmid DNA in donors and transconjugants

### 4.2.1 Extraction of plasmid DNA

Repeated attempts were made at extracting plasmid DNA from both the VanA GRE isolates and their corresponding transconjugants. Attempts with the RPM Spin Midi kit (BIO 101 Inc.) and the large-scale plasmid extraction method described by Heilig *et al.* (1998) were unsuccessful, failing to yield any plasmid DNA.

Potential plasmid DNA was, however, extracted by the technique described by Woodford *et al.* (1993), and so the technique was applied to a range of isolates, chosen so that many different PFGE types would be represented. Figure 4.1 (overleaf) shows the result of the plasmid extraction.

**Figure 4.1: Plasmid extractions performed according to the protocol described by Woodford *et al.* (1993)**




Lane: 1 - RIE-01; 2 - RIE-02; 3 - RIE-09; 4 - RIE-13; 5 - RIE-15; 6 - RIE-16; 7 - RIE-18; 8 - RIE-19; 9 - ECH-01; 10 - RIE-23; 11 - RIE-24; 12 - RIE-25; 13 - ECH-02; 14 - RIE-27; 15 - RIE-29; 16 - DNA ladder (λ*Hind*III)

As shown in figure 4.1, isolates deemed to be related by PFGE analysis gave rise to comparable bands following the plasmid extraction. Lanes 1-5 depict isolates of PFGE type A and related sub-types. Lanes 7 and 8 depict isolates that were identical by PFGE, as do lanes 10 and 11. The gel shown in figure 4.1 was subjected to alkali blotting, and the resulting membrane-bound DNA was probed for the presence of the *vanA* gene. The probe was generated from *vanA*-specific PCR product derived from primers *vanA1* and *vanA2* (table 2.3, page 66). The results of the *vanA* probing are illustrated in figure 4.2.

**Figure 4.2: *vanA*-probing of plasmid extraction depicted in figure 4.1.**

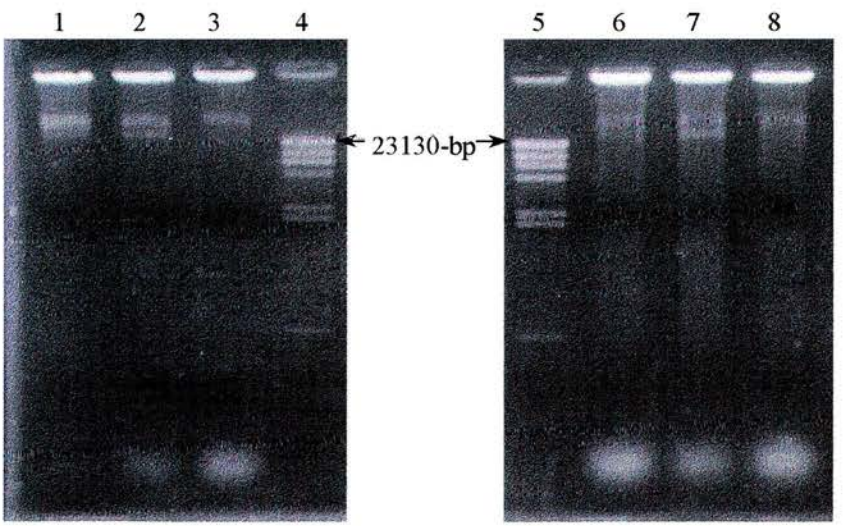

Lane: 1 - RIE-01; 2 - RIE-02; 3 - RIE-09; 4 - RIE-13; 5 - RIE-15; 6 - RIE-16; 7 - RIE-18; 8 - RIE-19; 9 - ECH-01; 10 - RIE-23; 11 - RIE-24; 12 - RIE-25; 13 - ECH-02; 14 - RIE-27; 15 - RIE-29. Molecular sizes indicated on figure 4.2 were estimated from the λ*Hind*III ladder that was run alongside samples (figure 4.1)

None of the presumed plasmid bands evident in figure 4.1 hybridized with the *vanA* probe. Indeed, many of the bands were recognizable as 'clear' bands amidst the high background signal present in many of the lanes, confirming that they were negative for *vanA*. Instead, all isolates, with the exception of ECH-02 (lane 13), had a common *vanA*-hybridizing band that was approximately 23-kb in size. This *vanA*-positive band was faint when compared with other bands present on the gel depicted in figure 4.1. Subsequent *vanA*-probing of the PFGE gels depicted in figure 3.2 revealed variation in the size of *vanA*-positive fragments present in some of those isolates depicted in figure 4.1 (see section 4.4.3). The contrasting picture illustrated

in figures 4.1 and 4.2, with a common *vanA*-positive band in all isolates, suggested that this band of 'plasmid' DNA was fragmented DNA, either chromosomal in nature, or from a large plasmid that had not survived the extraction procedure.

The same plasmid extraction protocol was applied to a selection of transconjugants and to the plasmid-free recipients used in the conjugation experiments. These attempts were unsuccessful, frequently yielding bands that were common to both the transconjugants and recipients (figure 4.3). These bands were most likely to be fragmented chromosomal DNA. Heavy chromosomal contamination was evident within the wells of the gel, and the results of *EcoR*I restriction of the plasmid extractions were consistent with a chromosomal restriction. Further attempts at plasmid extractions failed to yield plasmid DNA, including an alternative plasmid extraction method, specifically designed for the extraction of large plasmids (Crosa *et al.*, 1994)

**Figure 4.3: Plasmid extraction performed on transconjugants and recipients**




Lane: 1 - ARB-01; 2 - ARB-16; 3 - *E. faecium* GE-1; 4 - DNA ladder (λ*Hind*III); 5 - DNA ladder (λ*Hind*III); 6 - ARB-25; 7 - ARB-27; 8 - *E. faecalis* JH2-2.

### 4.2.2 Ethidium bromide curing of plasmid DNA

Ethidium bromide MICs were established for all 48 VanA isolates and transconjugants. *E. faecium* isolates were inhibited by ethidium bromide concentrations ranging from 60 to 70mg/L, whilst the MIC for *E. faecalis* isolates was typically less than 10mg/L.

Plasmid curing was performed on those isolates depicted in figure 4.1. Following overnight incubation in varying ethidium bromide concentrations, the highest concentration permitting visible growth was spread inoculated on a BHIA plate and the resulting colonies were screened for the loss of glycopeptide resistance. No loss of resistance was detected. Likewise, no loss of glycopeptide resistance occurred when plasmid curing was performed on a selection of transconjugant strains.

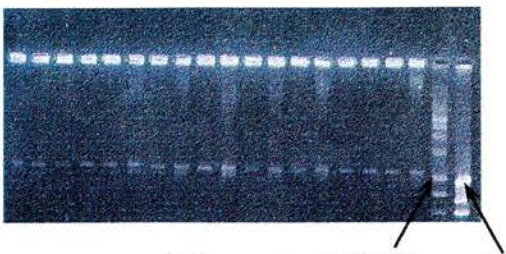
## 4.3 Detection of plasmids by electrophoresis of 'intact' genomic DNA

An alternative approach for the identification of plasmid DNA was adopted in view of the lack of success experienced with plasmid extractions. It was anticipated that electrophoresis of unrestricted PFGE plugs would enable the identification of plasmid DNA which would be free to migrate from the wells of a gel, as opposed to chromosomal DNA which would remain within the wells.

PFGE plugs were prepared for all 38 VanA donor strains that successfully transferred glycopeptide resistance and for the transconjugants arising from these donors. Plugs were also prepared for the two recipients used in the conjugation experiments. All donor strains were electrophoresed adjacent to their transconjugant. The resulting gels are depicted in figure 4.4.

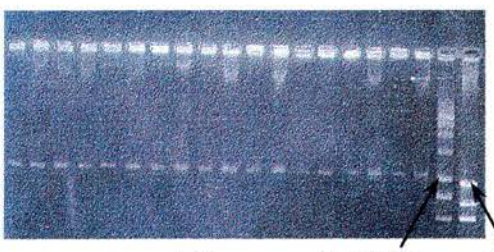
## Figure 4.4: Electrophoresis of 'intact' genomic DNA of donors and transconjugants

(a)




**Lane**: 1 - RIE-01; 2 - ARB-01; 3 - RIE-02; 4 - ARB-02; 5 - RIE-03; 6 - ARB-03; 7 - RIE-04; 8 - ARB-04; 9 - RIE-05; 10 - ARB-05; 11 - RIE-06; 12 - ARB-06; 13 - RIE-07; 14 - ARB-07; 15 - WGH-01; 16 - ARB-08; 17 - RIE-08; 18 - ARB-09; 19 - Super-coiled DNA ladder; 20 - λ*Hind*III DNA ladder

(b)

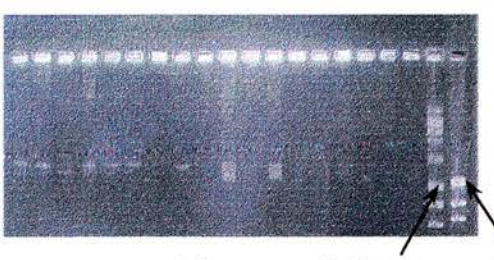



**Lane**: 1 - RIE-09; 2 - ARB-10; 3 - RIE-10; 4 - ARB-11; 5 - BGH-01; 6 - ARB-12; 7 - BGH-02; 8 - ARB-13; 9 - RIE-11; 10 - ARB-14; 11 - RIE-12; 12 - ARB-15; 13 - RIE-13; 14 - ARB-16; 15 - RIE-14; 16 - ARB-17; 17 - RIE-15; 18 - ARB-18; 19 - Super-coiled DNA ladder; 20 - λ*Hind*III DNA ladder

(c)

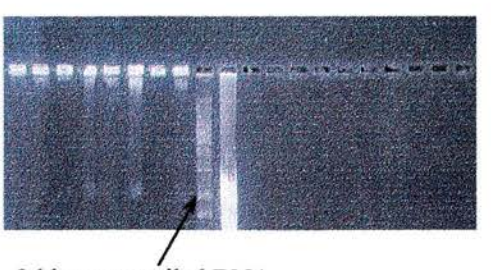

**Lane**: 1 - RIE-16; 2 - ARB-19; 3 - RIE-18; 4 - ARB-20; 5 - RIE-19; 6 - ARB-21; 7 - ECH-01; 8 - ARB-22; 9 - RIE-23; 10 - ARB-23; 11 - RIE-24; 12 - ARB-24; 13 - ECH-02; 14 - ARB-25; 15 - RIE-27; 16 - ARB-26; 17 - RIE-29; 18 - ARB-27; 19 - Super-coiled DNA ladder; 20 - λ*Hind*III DNA ladder

(d)




**Lane**: 1 - MON-01; 2 - ARB-28; 3 - ARI-01; 4 - ARB-29; 5 - RIE-30; 6 - ARB-30; 7 - ECH-01; 8 - ARB-22; 9 - NIN-03; 10 - ARB-31; 11 - NIN-04; 12 - ARB-32; 13 - GWI-06; 14 - ARB-38; 15 - GWI-07; 16 - ARB-33; 17 - GWI-08; 18 - ARB-34; 19 - Super-coiled DNA ladder; 20 - λ*Hind*III DNA ladder

(e)



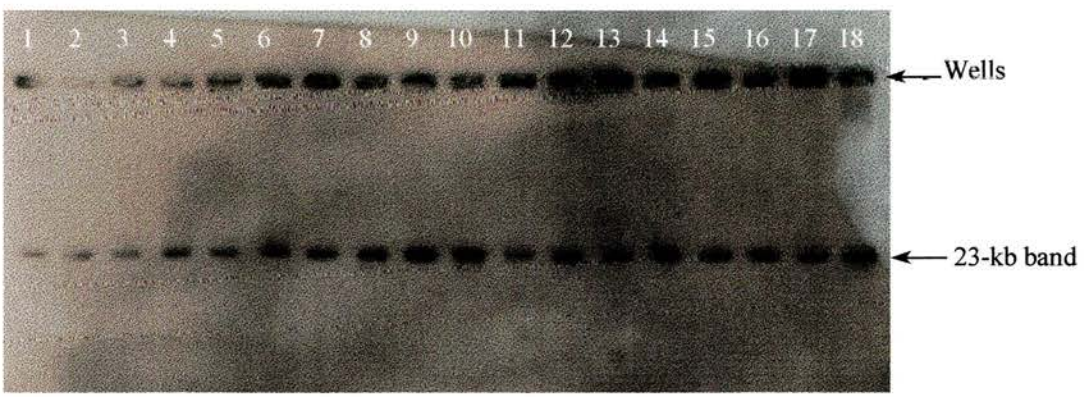

**Lane**: 1 - GWI-10; 2 - ARB-35; 3 - GWI-11; 4 - ARB-36; 5 - RIE-31; 6 - ARB-37; 7 - *E. faecium* GE-1; 8 - *E. faecalis* JH2-2; 9 - Super-coiled DNA ladder; 10 - λ*Hind*III DNA ladder

In figure 4.4 (a-e), lane numbers for all gels run from left to right.

The vast majority of strains (GRE donor strains and transconjugants) yielded a band of DNA equivalent to approximately 8-kb super-coiled DNA or 23-kb linear DNA, with this band occasionally replaced by a double band. All GRE isolates and transconjugants showed considerable DNA present within the wells of the gels. This was presumed to be chromosomal DNA. All gels were transferred to nylon membranes to enable probing for Tn*1546* sequences. A *vanR* probe was used, generated from *vanR*-specific PCR product obtained with the *vanR-f* and *vanR-r* primers (table 2.4, page 75).

Figure 4.5 shows the result of *vanR*-probing of the gel depicted in figure 4.4(a). As was typically the case for the other gels, probe hybridized to both DNA within the wells of the gel, and to the band equating to 23-kb linear DNA. All GRE donor strains and transconjugants were shown to be *vanR*-positive, with probe either hybridizing to the wells, bands within the gel, or both. The recipient strains (*E. faecium* GE-1 and *E. faecalis* JH2-2) did not hybridize with the *vanR* probe.

**Figure 4.5: *vanR*-probing of 'intact' genomic DNA**




Lane: 1 - RIE-01; 2 - ARB-01; 3 - RIE-02; 4 - ARB-02; 5 - RIE-03; 6 - ARB-03; 7 - RIE-04; 8 - ARB-04; 9 - RIE-05; 10 - ARB-05; 11 - RIE-06; 12 - ARB-06; 13 - RIE-07; 14 - ARB-07; 15 - WGH-01; 16 - ARB-08; 17 - RIE-08; 18 - ARB-09

Given that Tn*1546* is 10.8-kb in length, the predominant band identified as being *vanR*-positive (equating to 8-kb super-coiled DNA) was too small to be an intact plasmid harbouring the *van* gene cluster. It was likely, therefore, that this band had the same origin as the *vanA*-positive fragments seen in the plasmid extractions, being linear, fragmented DNA, either chromosomal or plasmid in nature.

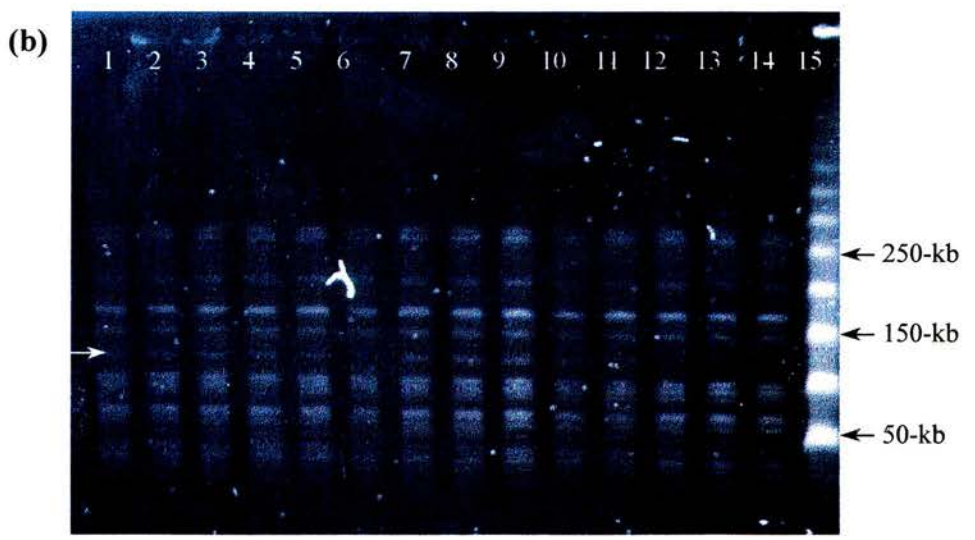
### 4.4 Probing of *Sma*I-restricted PFGE gels for Tn*1546*

All 48 VanA GRE isolates were analysed by PFGE, as described in section 3.1.2. The three PFGE gels, depicted in figure 3.2, were transferred to nylon membrane to facilitate probing for *vanA*. Likewise, the 37 transconjugants arising from conjugation experiments, listed in table 4.1, were subjected to PFGE analysis. This enabled identification of DNA fragments that were present within the transconjugants, but not within the recipients from which they were derived. It also facilitated *vanA* probing of the transconjugants to determine the location of Tn*1546*.

Figures 4.6 and 4.7 on the following pages show the PFGE gels of the transconjugants.

**Figure 4.6: PFGE analysis of *E. faecium* transconjugants**

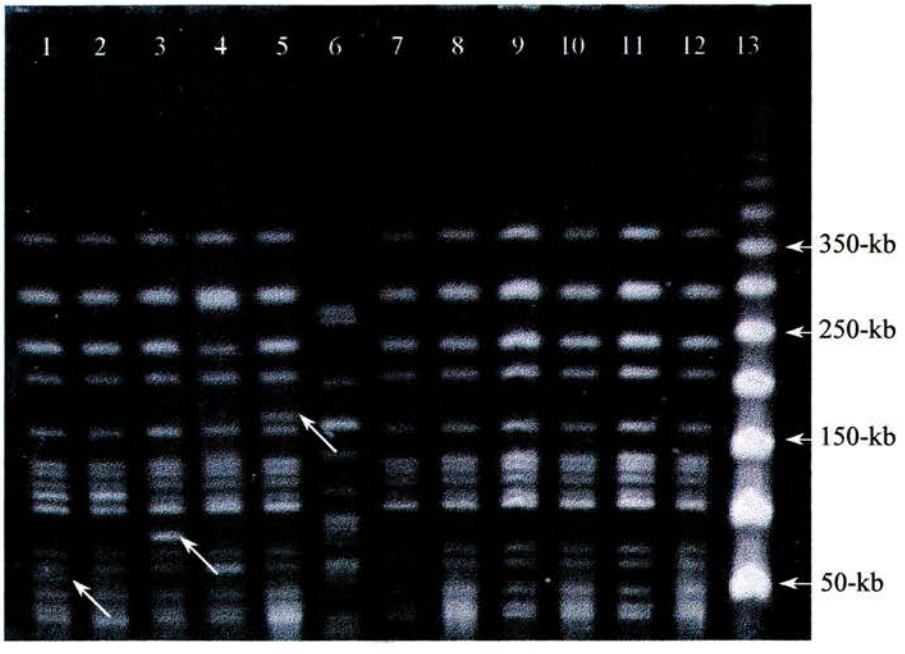

Lane: 1 - ARB-01; 2 - ARB-02; 3 - ARB-03; 4 - ARB-04; 5 - ARB-05; 6 - ARB-06; 7 - ARB-07; 8 - ARB-08; 9 - ARB-09; 10 - ARB-10; 11 - ARB-11; 12 - ARB-12; 13 - ARB-13; 14 - *E. faecium* GE-1; 15 - DNA ladder (50 - 1000 kb)




Lane: 1 - ARB-14; 2 - ARB-15; 3 - ARB-16; 4 - ARB-17; 5 - ARB-18; 6 - ARB-19; 7 - ARB-20; 8 - ARB-21; 9 - ARB-22; 10 - ARB-29; 11 - ARB-30; 12 - ARB-33; 13 - ARB-34; 14 - *E. faecium* GE-1; 15 - DNA ladder (50 - 1000 kb)

The white arrow in figures (a) and (b) indicate the position of the additional DNA fragment (see section 4.4.1).

**Figure 4.7: PFGE analysis of *E. faecalis* transconjugants**




Lane: 1 - ARB-23; 2 - ARB-24; 3 - ARB-25; 4 - ARB-26; 5 - ARB-27; (Lane 6 included in error); 7 - ARB-31; 8 - ARB-32; 9 - ARB-35; 10 - ARB-36; 11 - ARB-37; 12 - *E. faecalis* JH2-2; 13 - DNA ladder (50 - 1000 kb).

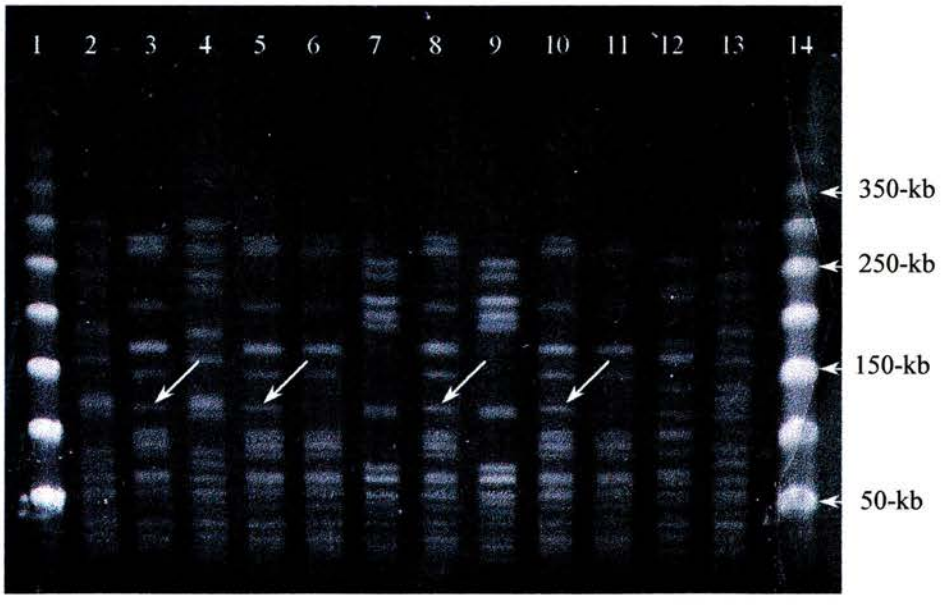
The white arrows indicate the positions of additional fragments (section 4.4.1).

### 4.4.1 PFGE analysis of transconjugants

As shown in figure 4.6, all *E. faecium* transconjugants, with the exception of ARB-33 and ARB-34 (derived from GWI-07 and GWI-08 respectively), displayed an additional DNA fragment within their PFGE pattern when compared with the PFGE pattern of *E. faecium* GE-1. This fragment was estimated at 115-kb in all transconjugants, with the exception of ARB-19, in which it appeared approximately 5-kb smaller. This suggested the presence of a common transferable genetic element in the majority of VanA *E. faecium* isolates from Edinburgh.

To establish if the same 115-kb fragment was present in the donor strains, a selection of donors and transconjugants were run alongside one another. Donor strains were chosen to include diverse strains (according to PFGE analysis), but all donor strains chosen gave rise to a transconjugant with the 115-kb additional fragment. As shown in figure 4.8, all donors studied had a DNA fragment within their PFGE pattern that matched the additional fragment seen in their transconjugant.

**Figure 4.8: PFGE analysis of selected donor strains alongside their corresponding transconjugant**




Lane: 1 - DNA ladder (50 - 1000 kb); 2 - RIE-03; 3 - ARB-03; 4 - RIE-04; 5 - ARB-04; 6 - *E. faecium* GE-1; 7 - RIE-18; 8 - ARB-20; 9 - RIE-19; 10 - ARB-21; 11 - *E. faecium* GE-1; 12 - ARI-01; 13 - RIE-30; 14 - DNA ladder (50 - 1000 kb).

The white arrows indicate the positions of the additional DNA fragments seen in transconjugants.

PFGE analysis of *E. faecalis* transconjugants (figure 4.7) showed only three to have clearly visible additional fragments when compared to the recipient strain, *E. faecalis* JH2-2. Transconjugant ARB-23 displayed an additional fragment of approximately 45-kb, ARB-25 had a fragment of approximately 75-kb, whilst ARB-27 had an additional fragment estimated at approximately172-kb. No other *E. faecalis* transconjugants displayed additional fragments within their PFGE patterns, although small-scale variation in the sizes of 'existing' fragments (those also present within the *E. faecalis* JH2-2 recipient) could not be ruled out.

### 4.4.2 Probing of transconjugant PFGE gels for Tn*1546*

The three gels depicted in figures 4.6 and 4.7 were probed for the presence of the *vanA* gene. All transconjugants that displayed an additional DNA fragment by PFGE analysis, as outlined in the previous section, yielded a single *vanA*-hybridizing fragment following probing of the PFGE gels. However, in all but one transconjugant, the size of the *vanA*-positive fragment did not match the size of the additional fragment that was seen in the PFGE pattern. Figure 4.9 shows the result of probing the gel depicted in figure 4.6(a) for the presence of the *vanA* gene. A common *vanA*-positive fragment was present in all transconjugants. The fragment was approximately 45-kb, some 70-kb smaller than the additional DNA fragment that was seen in the PFGE pattern of the transconjugants.

**Figure 4.9: *vanA*-probing of the transconjugant PFGE gel depicted in figure 4.6(a)**


Lane: 1 - ARB-01; 2 - ARB-02; 3 - ARB-03; 4 - ARB-04; 5 - ARB-05; 6 - ARB-06; 7 - ARB-07; 8 - ARB-08; 9 - ARB-09; 10 - ARB-10; 11 - ARB-11; 12 - ARB-12; 13 - ARB-13.
The sizes indicated were estimated from the DNA ladder shown in figure 4.6(a)

The only transconjugant to have a *vanA*-positive fragment of a size matching that of the additional fragment seen in its PFGE pattern was ARB-23. Both the additional and the *vanA*-positive fragments were estimated to be approximately 45-kb in size.

Of the 11 transconjugants that did not display an additional DNA fragment in their PFGE pattern, two yielded a *vanA*-positive fragment upon probing of the PFGE gel. Transconjugants ARB-24 and ARB-26 had a *vanA*-positive DNA fragment of approximately 38-kb and 53-kb respectively. Fragments in this size range were poorly resolved on the PFGE gels and so any small-scale variation in the sizes of such fragments would not have been detected. The results of the *vanA*-probing of transconjugant PFGE gels are summarized in table 4.2, alongside the results of probing the VanA donor strains, discussed in section 4.4.3.

## Table 4.2: Summary of the results of *vanA*-probing and PFGE analysis of transconjugants and VanA donor strains

| *vanA*-positive fragment in donor PFGE | | *vanA*-positive fragment in transconjugant PFGE | | Size of any additional fragment in transconjugant PFGE (kb) |
|---|---|---|---|---|
| **Donor** | **Size (kb)** | **Transconjugant** | **Size (kb)** | |
| RIE-01 | 45 | ARB-01 | 45 | 115 |
| RIE-02 | 45 | ARB-02 | 45 | 115 |
| RIE-03 | 45 | ARB-03 | 45 | 115 |
| RIE-04 | 45 | ARB-04 | 45 | 115 |
| RIE-05 | 45 | ARB-05 | 45 | 115 |
| RIE-06 | - | ARB-06 | 45 | 115 |
| RIE-07 | - | ARB-07 | 45 | 115 |
| WGH-01 | 45 | ARB-08 | 45 | 115 |
| RIE-08 | 45 | ARB-09 | 45 | 115 |
| RIE-09 | 45 | ARB-10 | 45 | 115 |
| RIE-10 | 45 | ARB-11 | 45 | 115 |
| BGH-01 | - | ARB-12 | 45 | 115 |
| BGH-02 | - | ARB-13 | 45 | 115 |
| RIE-11 | - | ARB-14 | 45 | 115 |
| RIE-12 | - | ARB-15 | 45 | 115 |
| RIE-13 | 45 | ARB-16 | 45 | 115 |
| RIE-14 | - | ARB-17 | 45 | 115 |
| RIE-15 | 45 | ARB-18 | 45 | 115 |
| RIE-16 | 45 | ARB-19 | 137 | 110 |
| RIE-18 | 60 | ARB-20 | 68 | 115 |
| RIE-19 | 60 | ARB-21 | 68 | 115 |
| ECH-01 | - | ARB-22 | 45 | 115 |
| RIE-23 | 38 | ARB-23 | 45 | 45 |
| RIE-24 | 38 | ARB-24 | 31 | - |
| ECH-02 | 115 | ARB-25 | 160 | 75 |
| RIE-27 | 38 | ARB-26 | 53 | - |
| RIE-29 | 38 | ARB-27 | 31 | 172 |
| MON-01 | - | ARB-28 | - | - |
| ARI-01 | - | ARB-29 | 45 | 115 |
| RIE-30 | 38 | ARB-30 | 45 | 115 |
| NIN-03 | - | ARB-31 | - | - |
| NIN-04 | - | ARB-32 | - | - |
| GWI-07 | - | ARB-33 | - | - |
| GWI-08 | - | ARB-34 | - | - |
| GWI-10 | - | ARB-35 | - | - |
| GWI-11 | - | ARB-36 | - | - |
| RIE-31 | - | ARB-37 | - | - |

'-' denotes that no *vanA* positive fragment was detected, or that no additional PFGE fragment was visible.

### 4.4.3 Probing of donor PFGE gels for Tn*1546*

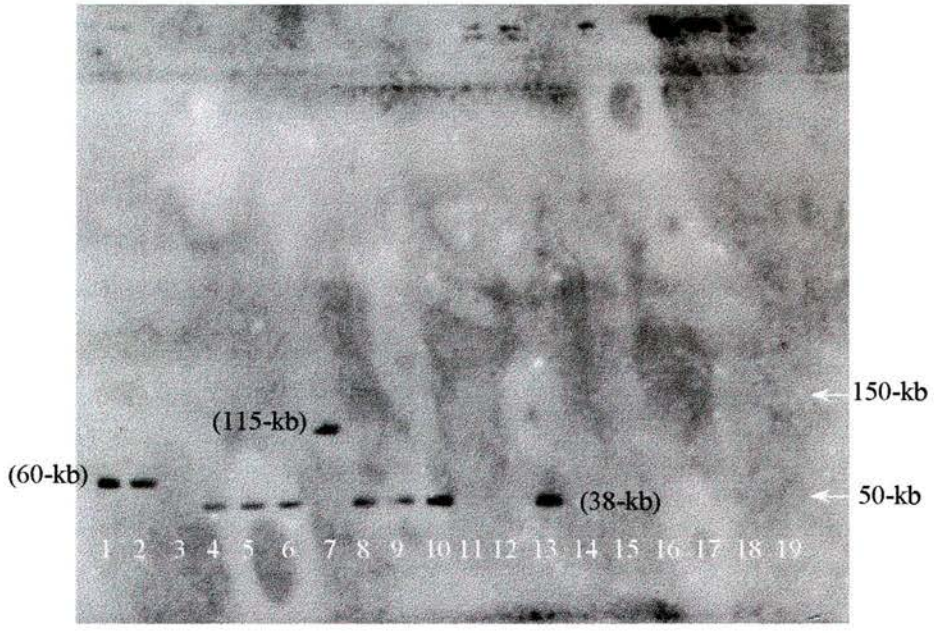
The three gels depicted in figure 3.2 were probed for the presence of the *vanA* gene, with typical hybridization results shown in figure 4.10 (overleaf). The PFGE gel depicted in figure 3.2(a) was run with a different DNA ladder from all the other gels. The ladder used (*Saccharomyces cerevisiae* DNA ladder) was not ideally suited for sizing of low molecular weight DNA fragments, as the smallest fragment in the DNA ladder is 225-kb. The size of the *vanA*-positive fragments detected from probing of this gel was estimated from a later gel, which was run with representatives of the isolates depicted in figure 3.2(a) alongside a suitable DNA ladder.

The results obtained from the *vanA*-probing of all gels are summarized in table 4.2. In brief, the majority of isolates belonging to the outbreak strain (PFGE type A) had a common *vanA*-positive fragment of approximately 45-kb. For many of these isolates, the signal generated by the *vanA* hybridization was very weak, and so members of the outbreak strain that did not appear to have a 45-kb fragment harbouring the *vanA* gene may simply have gone undetected. Isolates RIE-18 and RIE-19, identical by PFGE analysis, had a common *vanA*-positive fragment estimated as being 60-kb in size, whilst a group of clonally diverse isolates had a common *vanA*-positive band of approximately 38-kb (figure 4.10).

Table 4.2 only summarizes the *vanA*-probing results of those GRE isolates that successfully transferred glycopeptide resistance during conjugation. However, probing was performed on all VanA GRE isolates, including the ten isolates that failed to yield transconjugants. Of these ten isolates, three yielded a *vanA*-positive fragment upon hybridization. Two of these isolates (RIE-25 and RIE-26) were amongst the group of isolates displaying a *vanA*-positive fragment of approximately

38-kb depicted in figure 4.10. The remaining isolate, GWI-09, was found to have a *vanA*-positive fragment that was estimated at 204-kb.

**Figure 4.10: *vanA*-probing of the VanA donor PFGE gel depicted in figure 3.2(b)**




Lane: 1 - RIE-18; 2 - RIE-19; 3 - ECH-01; 4 - RIE-23; 5 - RIE-24; 6 - RIE-25; 7 - ECH-02; 8 - RIE-26; 9 - RIE-27; 10 - RIE-29; 11 - MON-01; 12 - MON-02; 13 - RIE-30; 14 - ARI-01; 15 - NIN-01; 16 - NIN-02; 17 - NIN-03; 18 - NIN-04; 19 - GWI-02. The sizes indicated were estimated from the DNA ladder shown in figure 3.2(b).

Analysis of the results presented in table 4.2 showed occasional disparity between the sizes of *vanA*-positive fragments that were seen in the VanA donor strains and those seen in the resulting transconjugants. In some cases, this may have been due to inaccurate sizing of the *vanA*-positive bands. As well as a visual estimation of band sizes, estimations were made using the Diversity Database software described previously, and thus the estimations were prone to the same difficulties that were

encountered when using Diversity Database to compare PFGE patterns. A single DNA ladder run in the last lane of a gel did not necessarily reflect the sizes of all DNA fragments in all lanes across the gel, owing to variation between lanes. It is therefore possible that when *vanA*-positive fragment sizes in donors and transconjugants differ only slightly, they may indeed be the same fragment. For example, isolates RIE-18 and RIE-19 both showed a 60-kb *vanA*-positive fragment, whilst their transconjugants (ARB-20 and ARB-21) had 68-kb *vanA*-positive fragments. In PFGE gels, there is insignificant difference in the migration distances of DNA fragments that differ by only 8-kb.

# Chapter 5: Results

## Diversity of Tn*1546*-related elements

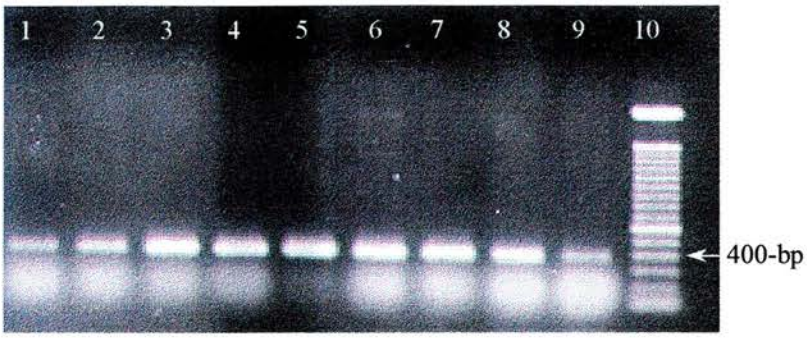
Prior to the application of long-range PCR (L-PCR) to amplify the entire length of Tn*1546*, shorter regions of the transposon were amplified by 'conventional' PCR to assess the diversity of the VanA elements within certain regions. Three different regions were examined: the *vanS-vanH*, *vanX-vanY* and *orf2-vanR* intergenic regions. These regions were chosen because variation, attributable to insertion and deletion events, had been described previously in all three regions. Sections 5.1 - 5.7 describe the PCR analysis performed on the 48 nosocomial VanA GRE. PCR analysis of the two VanA isolates from general practice specimens that were isolated during the course of the five-month epidemiology survey is discussed in brief in section 5.8.

## 5.1 PCR analysis of the *vanS-vanH* intergenic region

Handwerger *et al.* described a novel IS element, designated IS*1251*, within the *vanS-vanH* intergenic region of VanA enterococci isolated in the northeastern United States (Handwerger *et al.*, 1995). To screen for such variation within the VanA transposons of Scottish GRE, a 364-bp fragment, spanning the intergenic region, was amplified with the *vanS*-f and *vanH*-r primers listed in table 2.4.

All VanA GRE yielded a PCR amplicon of the anticipated size, as shown in figure 5.1. Restriction analysis of amplicons was performed with *Ssp*I restriction endonuclease, which cuts the prototype sequence once, generating two fragments (123-bp and 241-bp). All amplicons gave rise to the anticipated fragments upon restriction analysis, consistent with no insertion or deletion events within the *vanS-vanH* intergenic region.

**Figure 5.1: PCR analysis of the *vanS-vanH* intergenic region**




Lane: 1 - RIE-01; 2 - RIE-03; 3 - RIE-05; 4 - RIE-06; 5 - RIE-07; 6 - RIE-18; 7 - ECH-01; 8 - RIE-23; 9 - RIE-30; 10 - 100-bp DNA ladder

## 5.2 PCR analysis of the *vanX-vanY* intergenic region

Arthur *et al.* (1993) and Miele *et al.* (1995) both identified significant variation within the *vanX-vanY* intergenic region of certain Tn*1546*-related elements, although the nature of this variation was not established. Subsequently, many studies have shown that this variation is common and is most often attributable to the presence of IS*1216*-related insertion sequences. Often these insertions are accompanied by small-scale deletions. Primers *vanX*-f and *vanY*-r (table 2.4) were used to amplify the *vanX-vanY* intergenic region to detect any insertions or deletions present within the region.

Initially, few VanA isolates yielded any PCR amplicons, with those few yielding a prototype sized amplicon of 691-bp. Further success was achieved following optimization of the 'conventional' PCR protocol. However, it was not until L-PCR was adopted that all VanA isolates yielded a *vanX-vanY* PCR amplicon. Figure 5.2 shows the results of *vanX-vanY* PCR on a selection of VanA isolates. PCR

amplicons were either of the prototype size (691-bp) or in the region of 1500 base pairs. This increased amplicon size suggested the presence of an insertion of approximately 800 base pairs.

Initial restriction analysis was performed with *Xba*I restriction endonuclease, which cuts the prototype *vanX-vanY* amplicon once, generating fragments of 559 and 132-bp. Those amplicons of the prototype size yielded the anticipated restriction fragments upon *Xba*I restriction. All amplicons that were estimated to be approximately 1500-bp in size gave rise to a 132-bp fragment and a single fragment of approximately 1400-bp following *Xba*I restriction analysis.

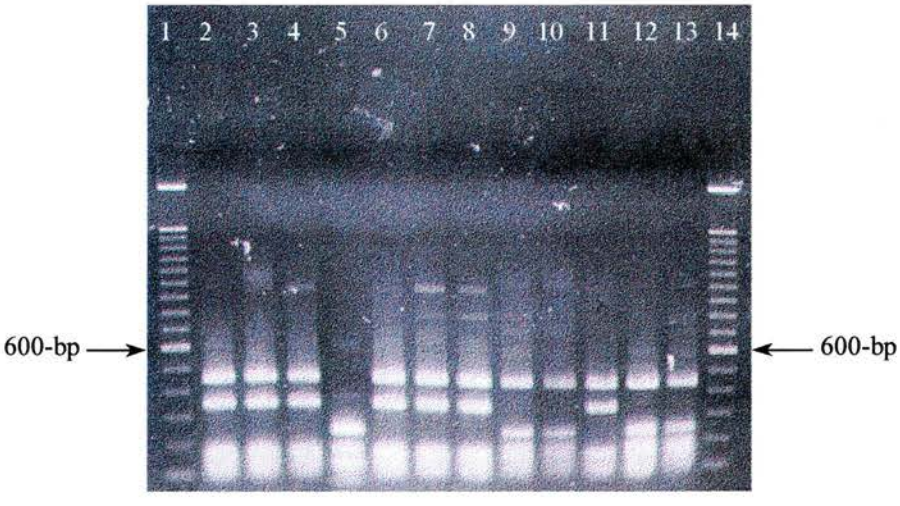
**Figure 5.2: PCR analysis of the *vanX-vanY* intergenic region**


Lane: 1 - BGH-01; 2 - BGH-02; 3 - ECH-01 (prototype amplicon); 4 - RIE-23; 5 - RIE-25; 6 - ECH-02; 7 - RIE-27; 8 - RIE-29; 9 - MON-01; 10 - MON-02; 11 - RIE-30; 12 - RIE-05; 13 - RIE-12; 14 - RIE-13; 15 - RIE-16; 16 - RIE-18; 17 - Negative control (*E. faecalis* ATCC 51299); 18 - Negative control (no template DNA); 19 - 100-bp DNA ladder.

As shown in figure 5.2, there appeared to be slight variation within the sizes of the amplicons that harboured the putative insertion. Further restriction analysis was

performed with *Mnl*I restriction endonuclease, a frequent cutter that would enable more detailed comparisons to be made. *Mnl*I cuts the prototype *vanX-vanY* amplicon four times, generating five fragments of 259, 184, 108, 96 and 44-bp. Figure 5.3 shows a selection of *vanX-vanY* amplicons following restriction with *Mnl*I.

**Figure 5.3: *Mnl*I restriction analysis of *vanX-vanY* PCR products**




Lane: 1 - 100-bp DNA ladder; 2 - RIE-05; 3 - BGH-01; 4 - RIE-18; 5 - ECH-01; 6 - RIE-23; 7 - RIE-25; 8 - RIE-29; 9 - MON-01; 10 - MON-02; 11 - RIE-30; 12 - GWI-07; 13 - GWI-06; 14 - 100-bp DNA ladder.

As shown in figure 5.3, *Mnl*I restriction showed three distinct restriction patterns. Lane 5 (ECH-01) showed the prototype *Mnl*I restriction pattern, consistent with no insertion or deletion events. Four of the five anticipated fragments were visible, with only the 44-bp fragment undetectable. A non-prototype restriction pattern (designated type 1) was evident in lanes 9, 10, 12 and 13. A different restriction pattern (designated type 2) was seen in lanes 2-4, 6-8 and 11. *Mnl*I restriction of the remaining non-prototype amplicons showed that all conformed to one of these two

non-prototype restriction profiles. Interestingly, the type 2 restriction profile of the *vanX-vanY* amplicon was found only in isolates from Edinburgh and the Borders (isolates with the three-letter prefix RIE, ECH, WGH or BGH). Of the 31 *vanA*-positive GRE from the Edinburgh/Borders region, 28 had a non-prototype *vanX-vanY* intergenic region of Tn*1546*. Twenty-six of these 28 displayed a type 2 *Mnl*I restriction profile, with the remaining two VanA elements displaying a type 1 restriction pattern. Only five of the 17 VanA GRE from outwith the Edinburgh/Borders region displayed a non-prototype *vanX-vanY* intergenic region. All five were shown to be type 1 by *Mnl*I restriction analysis.

Two non-prototype *vanX-vanY* amplicons were fully sequenced, one belonging to the type 1 *Mnl*I restriction group, the other to the type 2 restriction group. Initial sequencing was performed using the *vanX*-f and *vanY*-r primers that were used in the original PCR. On the basis of the partial sequence obtained, additional primers were designed to enable sequencing of the entire amplicon (primers 1216V-A, 1216V-B and 1216V-C; table 2.4). Sequencing showed that both amplicons harboured the same insertion sequence, showing greater than 99% homology to the IS*1216V*-like element described by Jensen (GenBank accession number AF093508; Jensen, 1998). The IS element is 809-bp in length and had inserted at nucleotide position 8839 (Tn*1546* co-ordinates) in both isolates sequenced. Insertion generated an 8-bp target site duplication. The sequence of both products was consistent with the IS*1216V*-like element being orientated so that transcription/translation occured in the opposite direction to that of the flanking genes.

The reason for the differing *Mnl*I restriction patterns of the two amplicons was ascertained as being a 93-bp deletion (nucleotides 8747-8839; Tn*1546* co-ordinates)

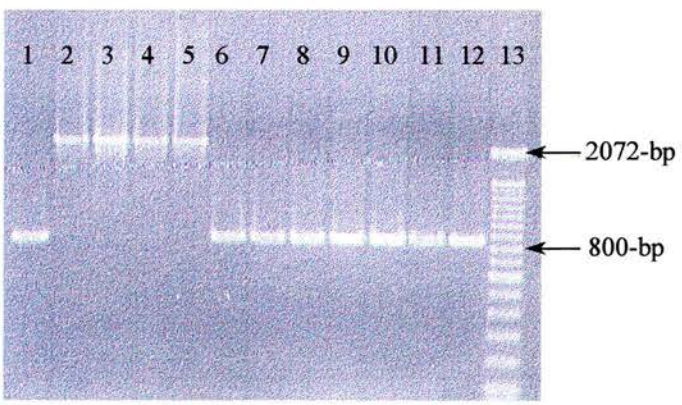
that had occurred immediately upstream of the IS*1216V*-like insertion point in the type 2 *vanX-vanY* amplicons. The deletion was within the *vanX-vanY* intergenic region and did not infringe on either the *vanX* or *vanY* gene sequences.

### 5.3 PCR analysis of the *orf2-vanR* intergenic region

The first description of variation within the *orf2-vanR* intergenic region was made by Woodford *et al.*, who identified the novel insertion sequence IS*1542* (Woodford *et al.*, 1998). All VanA GRE were thus examined for this, or other, insertions within the *orf2-vanR* region.

PCR was performed with primers *vanR*-f and *vanR*-r (table 2.4), designed to amplify an 859-bp fragment from within the *orf2-vanR* intergenic region to the *vanS* gene. Typical results from the *orf2-vanR* PCR are shown in figure 5.4.

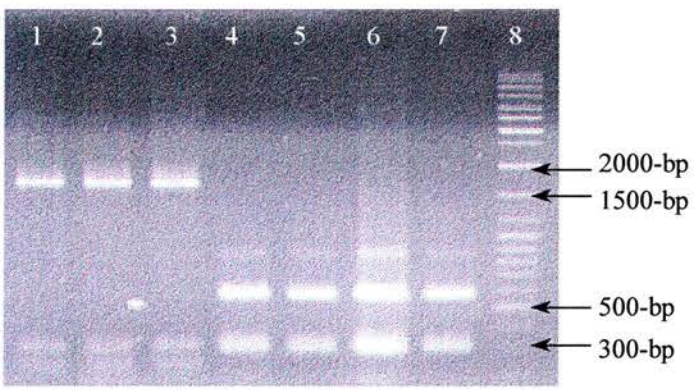
**Figure 5.4: PCR analysis of the *orf2-vanR* intergenic region**




Lane: 1 - ECH-01; 2 - RIE-27; 3 - RIE-29; 4 - MON-01; 5 - MON-02; 6 - RIE-31; 7 - NIN-01; 8 - NIN-02; 9 - NIN-03; 10 - NIN-04; 11 - GWI-02; 12 - GWI-03; 13 - 100-bp DNA ladder.

Two amplicon sizes were seen, as shown in figure 5.4. Of the 48 VanA GRE isolates, 15 yielded the anticipated amplicon size (859-bp). The remaining 33 isolates yielded an amplicon over 2-kb in size, consistent with an insertion of at least 1.2-kb. Interestingly, these 33 GRE isolates were the same 33 isolates previously identified as harbouring the IS*1216V*-like element within the *vanX-vanY* intergenic region. The *orf2-vanR* amplicons were subjected to restriction analysis with *Vsp*I restriction endonuclease. *Vsp*I should cut the prototype amplicon once, generating two fragments of 557 and 302-bp. This is shown in figure 5.5 (lanes 4-7), alongside the results of *Vsp*I restriction of three non-prototype amplicons (lanes 1-3).

**Figure 5.5: *Vsp*I restriction analysis of *orf2-vanR* PCR products**




Lane: 1 - GWI-06; 2 - GWI-07; 3 - GWI-08; 4 - GWI-09; 5 - GWI-10; 6 - GWI-11; 7 - RIE-31; 8 - GeneRuler DNA ladder mix (MBI Fermentas).

All non-prototype *orf2-vanR* amplicons yielded the same *Vsp*I restriction profile, displaying three bands of approximately 300, 250 and 1700-bp (lanes 1-3, figure 5.5). The insertion within the *orf2-vanR* region thus appeared to be the same in all isolates harbouring a non-prototype Tn*1546* element.

The amplicon of two non-prototype VanA isolates was sequenced to confirm the nature of the insertion. Initial sequencing was performed with the *vanR*-f and *vanR*-r primers. Whilst the sequence obtained from the *vanR*-r primer was solely that of the *vanR* gene, sequencing from the *vanR*-f primer yielded approximately 350 bases matching the published sequence of IS*1542* (GenBank accession number AF114715; Darini *et al.*, 1999). Based on the published sequence of IS*1542* and of Tn*1546*, two additional primers were designed (1542-A and 1542-B; table 2.4) to enable complete sequencing of the insert region. Sequencing confirmed the *orf2-vanR* insert within the 33 VanA isolates as being IS*1542*, displaying 100% identity with the published sequence of IS*1542*. The IS element was inserted at Tn*1546* nucleotide position 3932, the same position described by Woodford *et al.* (1998). As with the IS*1216V*-like element within the *vanX-vanY* intergenic region, IS*1542* was orientated so that transcription/translation occurs in the opposite direction to that of the flanking genes.

## 5.4 L-PCR analysis of Tn*1546*-related elements

As discussed in the previous sections, PCR analysis of specific regions within Tn*1546* had highlighted two IS elements within the majority of Tn*1546*-related elements: IS*1542* within the *orf2-vanR* intergenic region, and an IS*1216V*-like element within the *vanX-vanY* intergenic region. At this point it was decided to apply L-PCR with primer Tn*1546*-IR (table 2.4) to amplify the entire length of the VanA transposon. Initial attempts were generally unsuccessful, with few isolates yielding any PCR product. Those isolates that did yield PCR product were amongst the 15 isolates in which no variation from the prototype Tn*1546* had been found by previous PCR analysis. None of the 33 isolates previously identified as harbouring

IS*1542* and IS*1216V*-like insertions within their VanA transposon yielded L-PCR product with the Tn*1546*-IR primer. This raised the question of whether these non-prototype VanA elements had deletions within their 5' region, perhaps to compensate for the insertions elsewhere. If such deletions removed the left-hand inverted repeat, then one primer-annealing site would be lost.

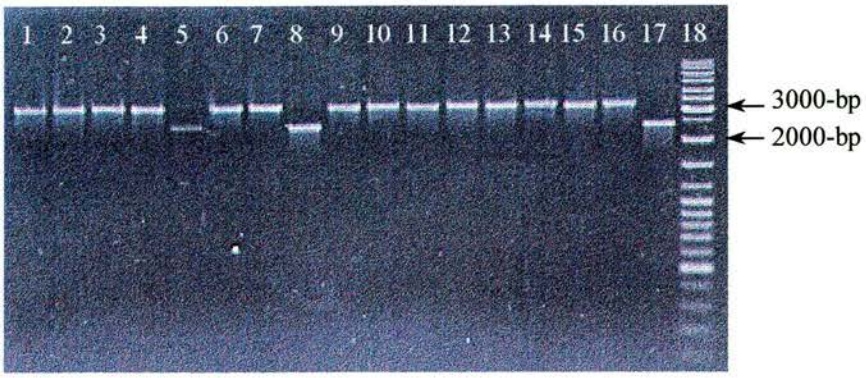
To assess the Tn*1546*-related elements for the presence of the left- and right-hand inverted repeats ($IR_L$ and $IR_R$ respectively), the Tn*1546*-IR primer was applied in PCR reactions with either the *vanR*-r primer (to amplify nucleotides 13-4699), or the *vanX*-f primer (to amplify nucleotides 8448-10839).

### 5.4.1 L-PCR analysis of the *vanX*-$IR_R$ region

All isolates studied yielded a PCR amplicon with the Tn*1546*-IR and *vanX*-f primers. The anticipated amplicon from a prototype Tn*1546* element is 2391-bp. The variation in the size of the L-PCR amplicons, as shown in figure 5.6, was consistent with the previous findings on variation within the *vanX-vanY* intergenic region. The presence of the IS*1216V*-like insertion increases the amplicon size to approximately 3.2-kb.

All *vanX*-$IR_R$ L-PCR amplicons were analysed by *Hinf*I restriction to establish if the non-essential *vanY* and *vanZ* genes contained any insertions or deletions. Table 5.1 shows the predicted *Hinf*I restriction fragments on the basis of (i) the known variation within the *vanX-vanY* intergenic region, and (ii) the published sequence of the *vanY* and *vanZ* genes (Genbank accession number M97297; Arthur *et al.*, 1993; Arthur *et al.*, 1995).

**Figure 5.6: L-PCR analysis of the *vanX*-$IR_R$ region**




Lane: 1 - RIE-03; 2 - WGH-01; 3 - BGH-01; 4 - BGH-02; 5 - RIE-14; 6 - RIE-18; 7 - RIE-19; 8 - ECH-01; 9 - RIE-25; 10 - ECH-02; 11 - RIE-26; 12 - RIE-27; 13 - RIE-29; 14 - MON-01; 15 - RIE-30; 16 - GWI-08; 17 - NIN-01; 18 - GeneRuler DNA ladder mix (MBI Fermentas).

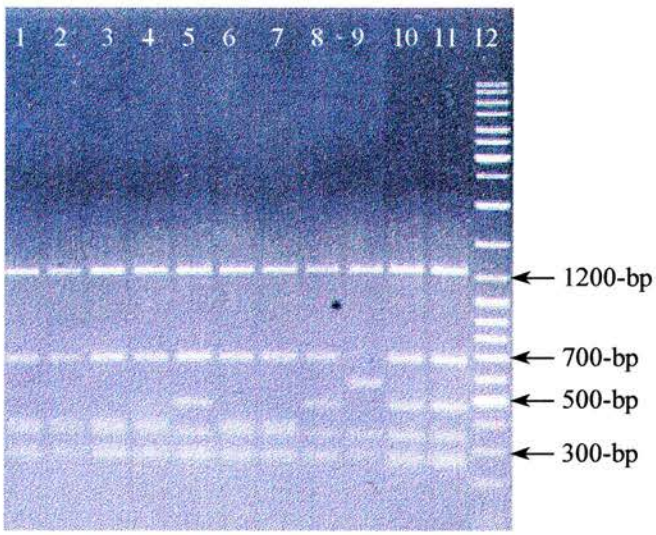
**Table 5.1: Anticipated *Hinf*I restriction fragments of *vanX*-$IR_R$ L-PCR amplicons**

| | **Anticipated *Hinf*I restriction fragments** |
|---|---|
| Prototype *vanX*-$IR_R$ sequence | 552, 1242, 261 & 337-bp |
| *vanX*-$IR_R$ harbouring IS*1216V*-like insertion | 362, 665, 248, 1242, 261 & 337-bp |
| *vanX*-$IR_R$ harbouring IS*1216V*-like insertion and 93-bp deletion | 455, 655, 248, 1242, 261 & 337-bp |

When the *Hinf*I restrictions were electrophoresed, the 337 and 362-bp fragments and the 248 and 261-bp fragments could not be distinguished from each other. The presence of the 665-bp fragment was indicative of the IS*1216V*-like element, whilst

the 455-bp fragment identified those amplicons which did not have the 93-bp deletion at the point of IS*1216V*-like insertion. Representative *Hinf*I restrictions are shown in figure 5.7. Lane 9 showed the *Hinf*I restriction typical of a prototype *vanX*-$IR_R$ sequence. The remaining lanes showed the *Hinf*I restriction of amplicons harbouring the IS*1216V*-like element, with the 93-bp deletion (lanes 1-4, 6 and 7), and without the deletion (lanes 5, 8, 10 and 11).

**Figure 5.7: *Hinf*I restriction analysis of *vanX*-$IR_R$ L-PCR products**




Lane: 1 - RIE-11; 2 - RIE-12; 3 - RIE-13; 4 - RIE-15; 5 - RIE-16; 6 - RIE-23; 7 - RIE-24; 8 - MON-02; 9 - ARI-01; 10 - GWI-07; 11 - GWI-08; 12 - GeneRuler DNA ladder mix (MBI Fermentas)

All isolates yielded the anticipated *Hinf*I restriction, and were thus confirmed as having the previously assigned *vanX-vanY* intergenic region, as well as intact *vanY* and *vanZ* genes.

### 5.4.2 PCR analysis of the 5' (*orf1*/*orf2*) region of Tn*1546*

#### 5.4.2.i PCR analysis internal to *orf1*

In contrast to the success of the *vanX*-$IR_R$ L-PCR, no isolates harbouring non-prototype Tn*1546*-related elements yielded a PCR product when studied by $IR_L$-*vanR* L-PCR. This supported the idea that perhaps the left-hand inverted repeat was absent in many, if not all, of the non-prototype VanA transposons.

To examine for large-scale deletions within *orf1*, attempts were made to amplify a 692-bp fragment internal to *orf1* with primers *orf1*-f and *orf1*-r (table 2.4). Of the 48 VanA GRE isolates, only five gave a non-prototype *orf1* amplicon. These five isolates (MON-01, MON-02, GWI-06, GWI-07 and GWI-08) all yielded a band of approximately 1.5-kb; all, but GWI-06, also yielded a prototype amplicon (692-bp). Restriction analysis of all *orf1* amplicons was performed with the *BsuR*I restriction endonuclease. The prototype amplicon is cut twice by *BsuR*I, generating fragments of 243, 129 and 320-bp. All amplicons of the prototype size yielded the anticipated restriction fragments. The 1.5-kb amplicon obtained from the five isolates listed above was inconsistent in its *BsuR*I restriction profile. Three of the five isolates (MON-01, GWI-07 and GWI-08) yielded two non-prototype fragments of approximately 600 and 900-bp following *BsuR*I restriction, whilst the 1.5-kb amplicon of MON-02 was not cut by *BsuR*I. No restriction pattern was visible for isolate GWI-06 due to the low yield of PCR amplicon. A selection of *BsuR*I restrictions is shown in figure 5.8.

If the 1.5-kb amplicon obtained from the five isolates listed above is a genuine PCR product, representing DNA between nucleotides 906 and 1597 (Tn*1546* co-ordinates; GenBank accession number M97297), it is interesting to note that the size

is consistent with there being an insertion of approximately 808-bp, thus matching the size of IS*1216V*-like elements. However, if such an insertion were responsible for the 1.5-kb amplicon, it would not be expected to simply yield two fragments upon *Bsu*RI restriction.

**Figure 5.8: *Bsu*RI restriction analysis of *orf1* PCR products**


Lane: 1 - GWI-02; 2 - GWI-03; 3 - GWI-04; 4 - GWI-05; 5 - GWI-06; 6 - GWI-07; 7 - GWI-08; 8 - GWI-09; 9 - GWI-10; 10 - GWI-11; 11 - RIE-31; 100-bp DNA ladder.

### 5.4.2.ii L-PCR analysis of the *orf1-vanR* region

The majority of isolates yielded a prototype 692-bp *orf1* amplicon. This suggested that if deletion events within non-prototype Tn*1546* elements had removed the $IR_L$, such deletions were restricted to the first 905-bp of the transposon. The PCR internal to *orf1* did not, however, give any indication of insertion or deletion events within the remainder of *orf1* (nucleotides 1598-3041) and within *orf2*.

L-PCR was thus employed with the *orf1*-f primer in combination with the *vanR*-r primer to amplify the region from nucleotides 906-4699. The anticipated amplicon

size from the *orf1-vanR* L-PCR is approximately 3.8-kb for those prototype Tn*1546* elements, and approximately 5.1-kb for those harbouring IS*1542* within the *orf2-vanR* intergenic region.

Seven of the 48 isolates failed to yield an *orf1-vanR* L-PCR amplicon, including isolates MON-01 and MON-02 that had previously shown non-prototype *orf1* amplicons. With the exception of isolates GWI-06 and GWI-07, all remaining VanA isolates yielded the anticipated L-PCR amplicon size, consistent with the presence or absence of IS*1542* as determined previously. All amplicons were subjected to restriction analysis with *Vsp*I restriction endonuclease. *Vsp*I cuts the prototype *orf1-vanR* sequence four times, generating five fragments of 757, 352, 1400, 982 and 303-bp in length. The IS*1542* insertion introduces another *Vsp*I cut site, thus giving rise to six fragments following *Vsp*I restriction. All isolates that had yielded a PCR amplicon of the prototype size (3.8-kb) yielded the appropriate restriction fragments following *Vsp*I restriction (lanes 14-19, figure 5.9). This was consistent with there being no variation between nucleotides 906 and 4699. Those isolates previously identified as harbouring the IS*1542* insertion (lanes 1-13, figure 5.9) showed no further variation (in the form of insertions and deletions) from the prototype sequence between nucleotides 906 and 4699. Thus the majority of isolates (39 of the 48) had no apparent insertions or deletions within nucleotides 906-3041 of *orf1*, or within the *orf2* gene. Interestingly, this group of 39 isolates included GWI-08, which had yielded two *orf1* amplicons, one of which was approximately 800-bp larger than anticipated (section 5.4.2.i). This potential variation within *orf1* was not detected by the *orf1-vanR* L-PCR.

**Figure 5.9: *Vsp*I restriction analysis of *orf1-vanR* L-PCR products**

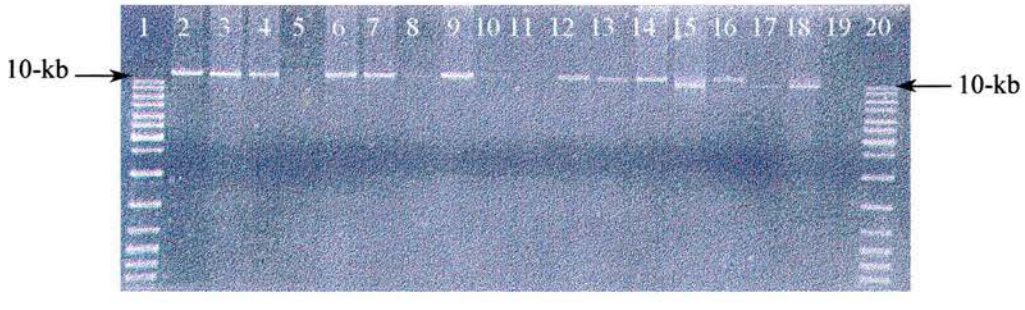

Lane: 1 - RIE-12; 2 - RIE-13; 3 - RIE-15; 4 - RIERIE-16; 5 - RIE-18; 6 - RIE-19; 7 - RIE-25; 8 - ECH-02; 9 - RIE-26; 10 - RIE-27; 11 - RIE-29; 12 - RIE-30; 13 - RIE-01; 14 - NIN-01; 15 - NIN-02; 16 - NIN-03; 17 - NIN-04; 18 - GWI-02; 19 - GWI-03; 20 - GeneRuler DNA ladder mix (MBI Fermentas)

GWI-06 and GWI-07 had also yielded *orf1* amplicons of approximately 1.5-kb, some 800-bp larger than anticipated. L-PCR analysis of the *orf1-vanR* region of these two isolates yielded two amplicons. One amplicon was consistent in size with the prototype Tn*1546* sequence (lacking the IS*1542* insertion), whilst the other amplicon appeared to be consistent with the presence of IS*1542*. Restriction analysis yielded multiple fragments, some of which fitted neither the pattern anticipated from restriction of the prototype amplicon, nor of the amplicon harbouring IS*1542*. The explanation for this result is not known. Both GWI-06 and GWI-07 had previously been identified as having IS*1542* within the *orf2-vanR* intergenic region by PCR and restriction analysis.

### 5.4.3 L-PCR analysis of the full-length Tn*1546*-related elements

Much of the above PCR analysis on the Tn*1546*-related elements was performed on the same genomic DNA extractions that were stored at -20°C. Some of those isolates that failed to yield *orf1-vanR* L-PCR amplicon were isolates that had previously been studied by L-PCR without failures. L-PCR requires template DNA that is relatively 'fresh', and thus it was decided to repeat the genomic extractions, so as to enable further L-PCR analysis of the VanA elements. The VanA isolates, having been stored at 4°C, were sub-cultured onto selective media prior to the genomic extractions. Initial attempts at full-length L-PCR with Tn*1546*-IR primer on the new genomic extractions yielded amplicon from the majority of isolates tested. L-PCR was repeated on the remaining isolates. The majority of isolates (43 of 48) yielded an amplicon of an appropriate size (>10-kb). Some size variation within the amplicons was seen, as shown in figure 5.10. This variation in size appeared to be consistent with the variation previously identified within the majority of VanA elements.

**Figure 5.10: L-PCR of Tn*1546*-related elements with Tn*1546*-IR primer**



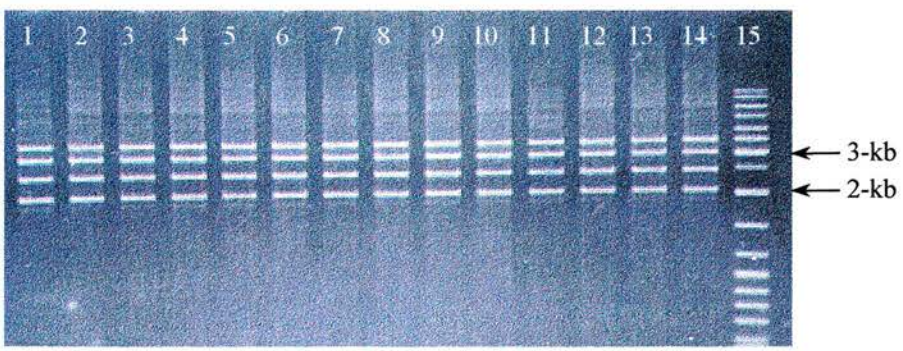

Lane: 1 - GeneRuler DNA ladder mix; 2 - RIE-16; 3 - RIE-18; 4 - RIE-19; 5 - ECH-01; 6 - RIE-23; 7 - RIE-24; 8 - RIE-25; 9 - ECH-02; 10 - RIE-26; 11 - RIE-27; 12 - RIE-29; 13 - MON-01; 14 - MON-02; 15 - ARI-01; 16 - RIE-30; 17 - NIN-01; 18 - NIN-02; 19 - Negative control (no template DNA); 20 - GeneRuler DNA ladder mix.

## 5.5 Restriction analysis of full-length Tn*1546*-related PCR amplicons

*Cla*I restriction analysis was performed on the 43 Tn*1546* L-PCR amplicons. Prior to restriction analysis, the sizes of *Cla*I restriction fragments were predicted on the basis of the known variation within the Tn*1546*-related elements. As shown in figure 5.11 (following page), *Cla*I cuts the prototype Tn*1546* element in three places, generating four fragments. Both IS*1542* and the IS*1216V*-like element introduce an additional *Cla*I cut site, thus generating six fragments, assuming no further variation is present elsewhere within the transposon.

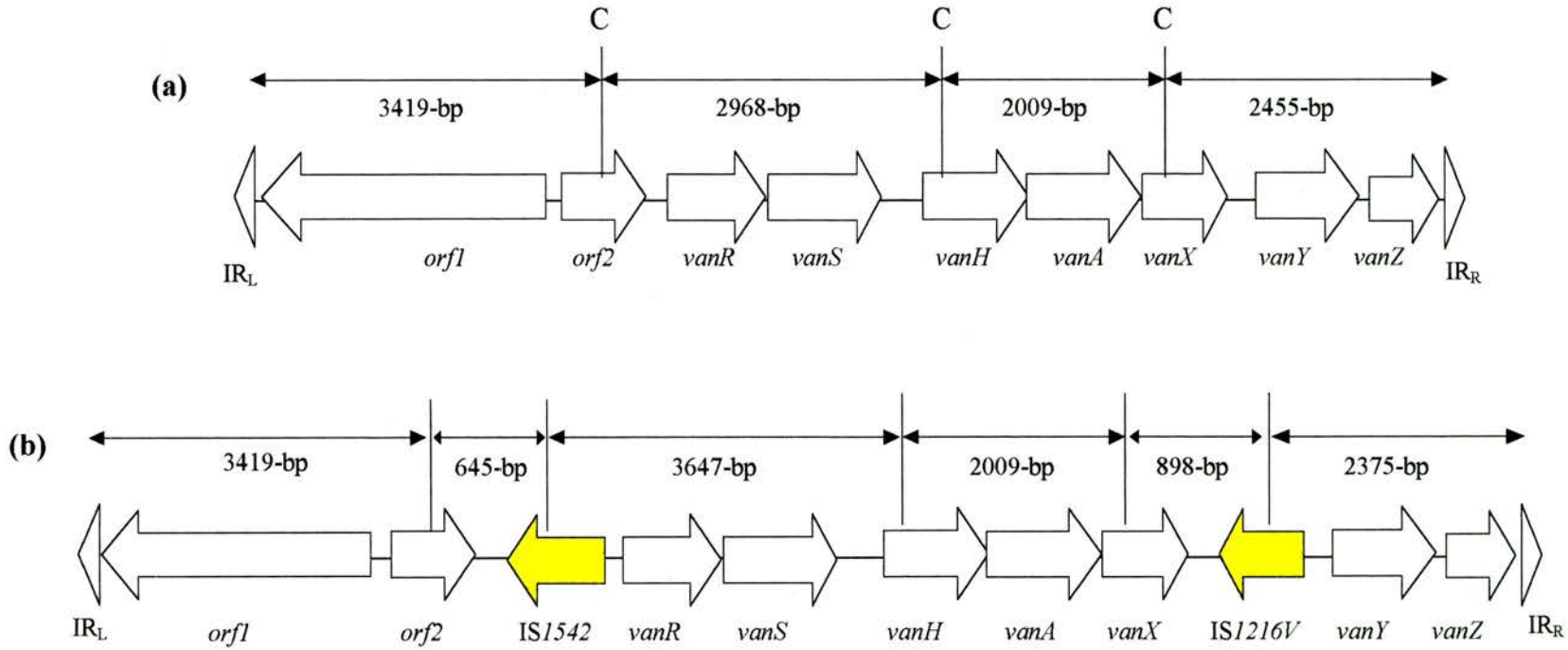
Figure 5.12 shows the *Cla*I restriction of prototype Tn*1546* elements, confirming 14 of the 48 isolates harboured a prototype VanA transposon, designated Tn*1546* type A. A further nine transposon types (B-K) were identified on the basis of their *Cla*I restriction profile. The yield of non-prototype L-PCR amplicon was extremely variable and so their *Cla*I restriction patterns (shown in figure 5.13) were not all readily visible. To enable easier comparison of Tn*1546* types, figure 5.14 is a diagrammatic representation of the different *Cla*I restriction patterns that were identified amongst the 48 isolates studied.

**Figure 5.12: *Cla*I restriction analysis of prototype Tn*1546* elements**



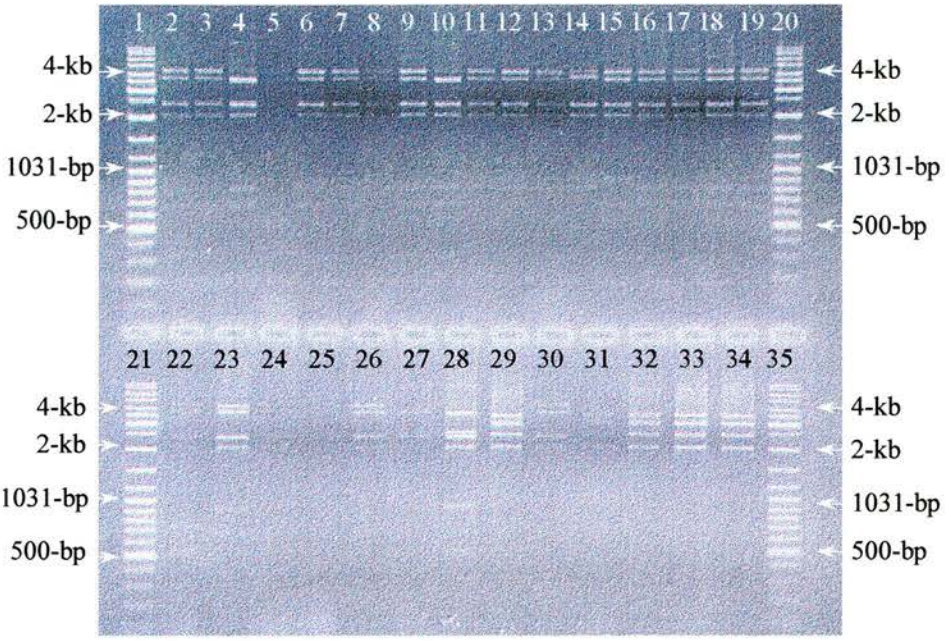

Lane: 1 - ECH-01; 2 - ARI-01; 3 - NIN-01; 4 - NIN-02; 5 - NIN-03; 6 - NIN-04; 7 - GWI-02; 8 - GWI-03; 9 - GWI-04; 10 - GWI-05; 11 - GWI-09; 12 - GWI-10; 13 - GWI-11; 14 - RIE-31; 15 - GeneRuler DNA ladder mix.

**Figure 5.11: *Cla*I restriction maps of (a) the prototype Tn*1546* element, and (b) the non-prototype element harbouring IS*1542* and IS*1216V*-like insertion sequences**




The 93-bp deletion that is present in the majority of isolates immediately adjacent to the point of insertion of the IS*1216V*-like element will reduce the size of the 898-bp fragment to 805-bp.

**Figure 5.13: *Cla*I restriction analysis of Tn*1546*-related elements**




Lane: 1 - GeneRuler DNA ladder mix; 2 - RIE-01; 3 - RIE-02; 4 - RIE-03; 5 - RIE-04; 6 - RIE-05; 7 - RIE-06; 8 - RIE-07; 9 - WGH-01; 10 - RIE-08; 11 - RIE-09; 12 - RIE-10; 13 - BGH-02; 14 - RIE-11; 15 - RIE-16; 16 - RIE-18; 17 - RIE-19; 18 - RIE-23; 19 - RIE-24; 20 - GeneRuler DNA ladder mix.

Lane: 21 - GeneRuler DNA ladder mix; 22 - RIE-25; 23 - ECH-02; 24 - RIE-26; 25 - RIE-27; 26 - RIE-29; 27 - MON-01; 28 - MON-02; 29 - ARI-01; 30 - RIE-30; 31 - NIN-01; 32 - NIN-02; 33 - NIN-03; 34 - NIN-04; 35 - GeneRuler DNA ladder mix.

None of the isolates previously identified as harbouring the IS*1542* and IS*1216V*-like insertions displayed the *Cla*I restriction profile predicted in figure 5.11, thus indicating that all isolates had variation elsewhere within the VanA transposon. The bands depicted in yellow in figure 5.14 (on following page) are those that were not predicted and thus represent further variation. Table 5.2 lists those isolates displaying each of the Tn*1546* types identified in figure 5.14.

**Figure 5.14: Diagrammatic representation of Tn*1546* types A-K on the basis of their *Cla*I restriction profiles**

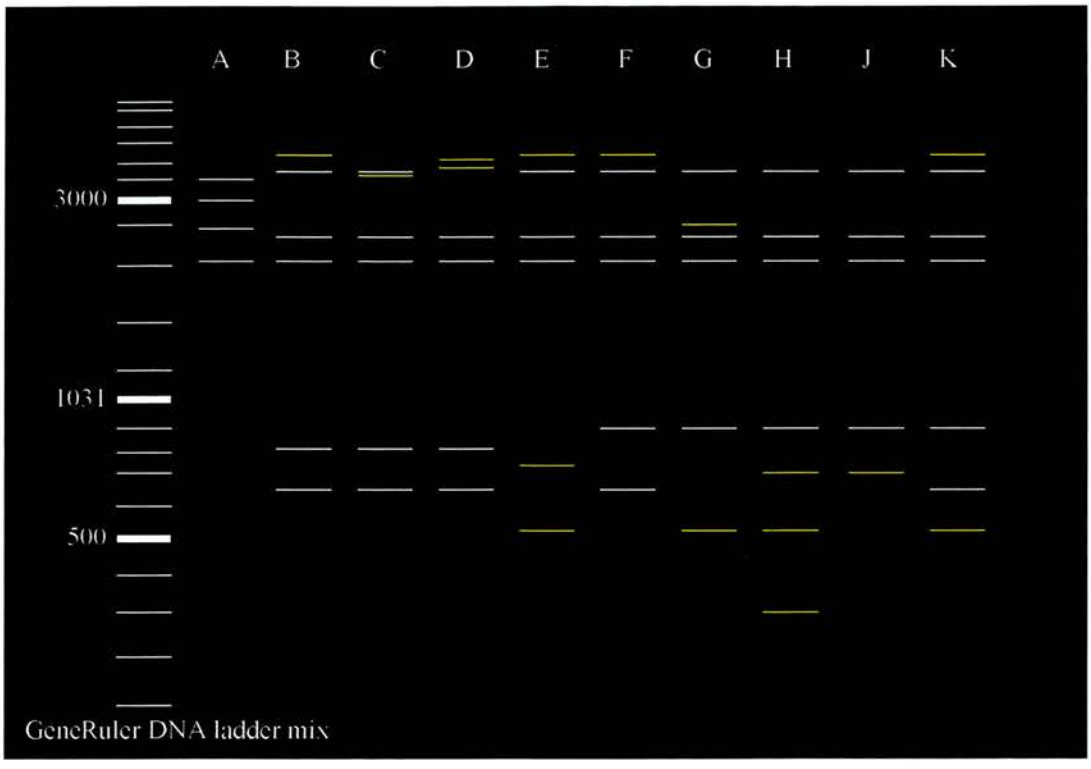



**Table 5.2: VanA isolates harbouring the Tn*1546* types defined in figure 5.14.**

| Tn*1546* type | Number | Isolates |
|---|---|---|
| A | 14 | ECH-01, ARI-01, NIN-01, NIN-02, NIN-03, NIN-04, GWI-02, GWI-03, GWI-04, GWI-05, GWI-09, GWI-10, GWI-11, RIE-31 |
| B | 14 | RIE-01, RIE-02, RIE-05, RIE-06, RIE-07, WGH-01, RIE-09, RIE-10, BGH-02, RIE-18, RIE-19, RIE-23, RIE-24, RIE-30 |
| C | 2 | RIE-03, RIE-08 |
| D | 1 | RIE-11 |
| E | 1 | RIE-25 |
| F | 1 | ECH-02 |
| G | 2 | MON-01, MON-02 |
| H | 1 | GWI-07 |
| J | 2 | GWI-06, GWI-08 |
| K | 1 | RIE-16 |

Tn*1546* type A is the prototype transposon

Thirty-nine of the 48 VanA isolates are listed in table 5.2. The remaining nine isolates could not be assigned to a Tn*1546* type on the basis of their *Cla*I restriction profile. Five of these isolates (BGH-01, RIE-12, RIE-13, RIE-14 and RIE-15) failed to yield an L-PCR amplicon despite repeated attempts. The remaining isolates (RIE-04, RIE-26, RIE-27 and RIE-29) yielded non-prototype L-PCR amplicon, but the yield of L-PCR product was too low to permit restriction analysis.

Of the VanA isolates that harboured a non-prototype Tn*1546*-related element, approximately half harboured a type B transposon. The *Cla*I restriction profile of the type B transposon varied from the predicted profile (based on the known IS*1542* and IS*1216V*-like insertions) solely on the size of the 3419-bp fragment of the *orf1/orf2* region (figure 5.11). The 3419-bp fragment was replaced by a fragment approximately 4150-bp in size, suggesting a possible insertion within the *orf1/orf2* region. On the basis of previous PCR results, this putative insertion was thought to be within the first 905-bp of the VanA transposon. Tn*1546* type F, displayed by only one isolate (ECH-02), had the same variation within the 3419-bp fragment as that seen in the type B transposon. The only difference between the transposon types B and F was the 93-bp deletion within the *vanX-vanY* intergenic region of the type B transposons.

Variation of transposon type K from the predicted *Cla*I restriction profile was also due to variation within the *orf1/orf2* region, altering the size of the 3419-bp fragment. The restriction profile of transposon type K was the same as that for the type F transposon, with the exception of an additional band of approximately 500-bp.

Transposon type C also varied from the predicted *Cla*I restriction profile solely by the size of the 3419-bp fragment. However, the fragment appeared only marginally

larger than predicted, at just over 3.5-kb. This increase in size was too small to be caused by the presence of an intact insertion sequence alone, and may have reflected the insertion of an IS element followed by a large-scale deletion of a size approximately 100-bp less than that of the IS element.

Earlier PCR analysis performed on isolates MON-01 and MON-02 suggested a possible insertion within the *orf1* gene (see section 5.4.2.i), but no L-PCR amplicon was obtained from either isolate with the *orf1*-f/*vanR*-r primers. These results suggested possible variation within the *orf1*/*orf2* region, and *Cla*I analysis of the Tn*1546* L-PCR amplicons of both isolates supported this suggestion. The isolates (assigned to Tn*1546* type G) both appeared to have substantial deletions within the *orf1*/*orf2* region. Their *Cla*I restriction profiles were missing both the 3419 and 645-bp fragments, which correspond to the *orf1*/*orf2* region. Instead, fragments of approximately 2500 and 500-bp were apparent, suggesting a deletion of approximately 1-kb. The size of this deletion may have been larger than this if it had followed the insertion of an IS element (see section 5.6).

The three VanA isolates from Glasgow's Western Infirmary identified as harbouring non-prototype VanA elements (GWI-06, GWI-07 and GWI-08) also appeared to have substantial deletions within the *orf1*/*orf2* region. Like isolates MON-01 and MON-02, these three Glasgow isolates yielded an unexpected *orf1* amplicon, albeit in addition to the prototype *orf1* amplicon (section 5.4.2.i). In addition, results from the *orf1*-f/*vanR*-r L-PCR on these three isolates were inconsistent with other PCR analysis performed previously. The *Cla*I restriction profiles of the Tn*1546* elements of the three isolates (types H and J) were both missing the 3419-bp fragment corresponding to *orf1*/*orf2* and the 645-bp fragment

generated by the additional *Cla*I site that is introduced by IS*1542*. In the place of these two bands, Tn*1546* type H (GWI-07) had three fragments of approximately 700, 500 and 300-bp. These three fragments combined amount to approximately 2.5-kb less than the two missing fragments. Tn*1546* type J (GWI-06 and GWI-08) showed the same 700-bp fragment that was seen in type H, although the 500 and 300-bp fragments were missing. However, the 700-bp fragment of type J was very faint, so it is possible that the 500 and 300-bp fragments have simply gone undetected. The significance of these findings in relation to the previous PCR analysis performed on these three isolates is unclear. There certainly appears to be significant variation within the *orf1*/*orf2* region. It may be that some of this variation arose during cold storage of the isolates, after the initial PCR analysis was performed.

It is certainly thought that isolate RIE-25, displaying Tn*1546* type E, underwent Tn*1546* variation during cold storage. As in other non-prototype Tn*1546* elements, the anticipated 3419-bp fragment was absent. However, also missing from Tn*1546* type E were the 645 and 805-bp fragments, indicative of the previously identified IS*1542* and IS*1216V*-like insertions respectively. No attempts were made to ascertain the nature of this variation.

Finally, Tn*1546* type D, harboured by isolate RIE-11, appeared to have variation located outwith the *orf1*/*orf2* region other than that identified by earlier PCR analysis. In addition to an alteration within the 3419-bp *orf1*/*orf2* fragment, the predicted 3647-bp fragment also appeared to be altered. The two bands were, instead, just under and just over 4-kb in size. It was unclear which fragment corresponded to which region of the transposon. Both the 645-bp and the 2009-bp

fragments that were predicted (figure 5.11) were unaffected by the variation, and the isolate had previously yielded a prototype amplicon from the *vanS-vanH* intergenic region. If such variation was indeed present, it either suggested that the isolate had undergone variation during cold storage, perhaps within the *vanS-vanH* intergenic region, or alternatively that the *vanS* gene may have been disrupted by an insertion.

### 5.6 Hybridization analysis of Tn*1546* types A-J

*Cla*I and *BamH*I restrictions were performed with representatives of Tn*1546* types A-J. Tn*1546* type K was not recognized until later, and was thus not included. The restrictions were electrophoresed and transferred to nylon membranes to facilitate probing.

#### 5.6.1 Probing of *Cla*I restrictions for the *vanA* gene

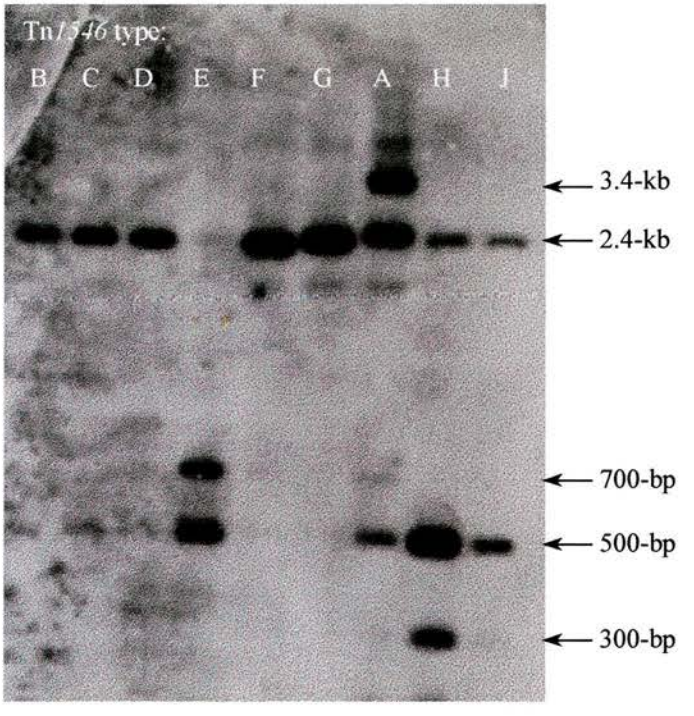
As a way of confirming that all L-PCR amplicons obtained with Tn*1546*-IR primer were, indeed, Tn*1546*-derived, the *Cla*I restrictions were probed for the presence of the *vanA* gene. A common fragment was found to be *vanA*-positive in all Tn*1546* types studied, the fragment estimated as being 2-kb in size. This corresponded to the 2009-bp fragment within the *vanHAX* genes that is obtained by *Cla*I restriction analysis (figure 5.11). All L-PCR amplicons were thus Tn*1546*-derived.

#### 5.6.2 Probing of *Cla*I restrictions for the inverted repeat (IR)

To try and identify those *Cla*I restriction fragments corresponding to the extreme 5' and 3' ends of the transposon, thus containing the left and right-hand inverted

repeats, the Tn*1546*-IR primer was fluorescein-labelled and used as an oligonucleotide probe. The same membrane that was probed for the *vanA* gene was probed for the inverted repeat. As shown in figure 5.11, a prototype Tn*1546* element would be expected to have IR-positive fragments of 3419 and 2455-bp following *Cla*I restriction. Those Tn*1546*-related elements that harbour the IS*1216V*-like insertion within the *vanX-vanY* intergenic region should have a 2375-bp fragment containing the right-hand inverted repeat. The size of the fragment harbouring the left-hand inverted repeat would be expected to vary according to the nature of the variation within the *orf1/orf2* region. The results of the IR-probing are shown in figure 5.15.

**Figure 5.15: IR-probing of *Cla*I-restricted Tn*1546* types A-J**



In an attempt to compensate for the low yield of L-PCR amplicon obtained from many of the isolates, the amount of amplicon restricted, and subsequently electrophoresed, was higher than that which would normally be used. It was thought that this 'over-loading' of lanes was responsible for some spurious IR-hybridizing bands that are evident in figure 5.15.

The prototype Tn*1546* element in figure 5.15 (type A) showed IR-positive fragments corresponding to *Cla*I restriction fragments of 3419 and 2455-bp as anticipated. Other IR-positive fragments visible in the restriction of the type A transposon did not match with any restriction fragments of that transposon, and were probably caused by overloading of lanes and perhaps spillage from neighbouring wells when loading samples into the gel.

With the exception of Tn*1546* type E, all other non-prototype Tn*1546* types showed an IR-positive fragment corresponding to the anticipated *Cla*I restriction fragment of 2375-bp, which contains the right-hand inverted repeat. Unexpectedly, however, no other IR-positive fragments were seen in Tn*1546* types B, C, D, F and G. Tn*1546* types H and J both showed other IR-positive fragments besides the 2375-bp fragment. Both the 500 and 300-bp fragment seen in the *Cla*I restriction of Tn*1546* type H (figure 5.14) appeared to be IR-positive. It was suggested previously that the lack of 500 and 300-bp fragments in Tn*1546* type J might simply be due to them being undetected. In support of this theory, Tn*1546* type J showed an IR-positive fragment of 500-bp. However, given that overflow from the well of lane 8 (Tn*1546* type H) may account for the IR-positive 500-bp fragment seen in lane 7 (Tn*1546* type A), the 500-bp IR-positive fragment seen in Tn*1546* type J may have a similar source.

Finally, the previous suggestion that isolate RIE-25, displaying Tn*1546* type E, had undergone considerable alteration within the Tn*1546* element during cold storage was supported by the results of IR-probing. As shown in figure 5.15, the only IR-positive fragments corresponded to the 700 and 500-bp fragments seen within the *Cla*I restriction profile (figure 5.14). Despite the *Cla*I restriction yielding a fragment apparently matching the predicted 2375-bp fragment, consistent with the *vanYZ* region at the 3' end of the transposon, this fragment did not hybridize with the IR probe.

The failure of many Tn*1546* types to yield two IR-positive fragments may be explained by the left-hand inverted repeat having undergone alteration. This may also partly explain the difficulties experienced in L-PCR with the Tn*1546*-IR primer. Clearly, however, the inverted repeat must be present in some form, otherwise no L-PCR amplicon would have been obtained. Decreasing the stringency of the hybridization process failed to identify any other IR-positive fragments. An alternative, and perhaps more plausible, explanation for the single IR-positive fragment is that an inverted duplication of the 3' end of Tn*1546* has replaced part of *orf1*. This possibility is discussed in section 7.4.

### 5.6.3 Probing of *BamH*I restrictions for the IS*1216V*-like element

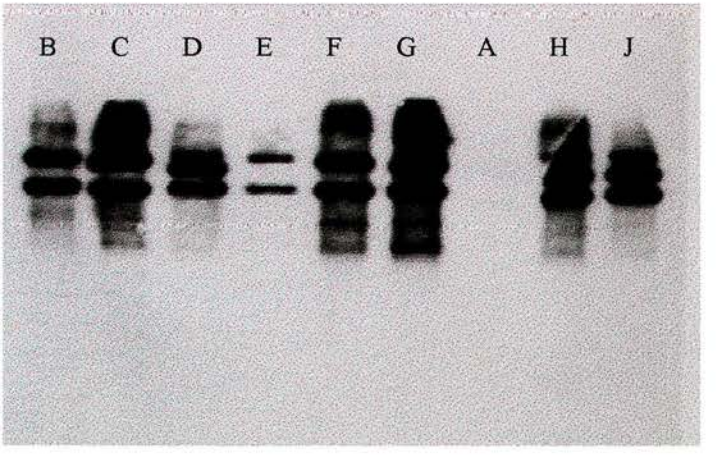
Given the unknown nature of the variation within the *orf1/orf2* region of the majority of the Tn*1546*-related elements identified, it was decided to probe this region for the presence of an IS*1216V*-like element, like that seen in the *vanX-vanY* intergenic region. An IS*1216V*-like probe was generated from the PCR product obtained with primers 1216V-D and 1216V-E (table 2.4). Tn*1546*-related elements

(representing types A-J) were *BamH*I-restricted. *BamH*I was chosen as it cuts the Tn*1546* element once within the *vanA* gene, and thus separates the known IS*1216V*-like element within the *vanX-vanY* intergenic region from the unknown variation within the *orf1/orf2* region. Figure 5.16 shows the result of the *BamH*I restriction, with the result of the IS*1216V*-probing shown in figure 5.17.

**Figure 5.16: *BamH*I restriction analysis of Tn*1546* types A-J**


DNA ladder is the GeneRuler DNA ladder mix.

**Figure 5.17: IS*1216V*-like probing of *BamH*I-restricted Tn*1546* types A-J**




For approximate sizes of IS*1216V*-positive fragments, refer to figure 5.16.

Again, over-loading of the lanes to compensate for low PCR yield led to 'dirty' hybridization results. Neither of the fragments generated by the *BamH*I restriction of the prototype Tn*1546* element hybridized with the IS*1216V*-like probe. However, all non-prototype Tn*1546* elements had two fragments which hybridized with the probe for the IS*1216V*-like insertion sequence. These results indicated that an IS element related to the IS*1216V*-like element was present within the 5' region of Tn*1546* types B-J. It should be noted, however, that the *Cla*I restriction of Tn*1546* types B, C, F and perhaps J only had one fragment of altered size, rather than additional fragments. The IS*1216V*-like insertion identified within the *vanX-vanY* intergenic region has a *Cla*I cut site, and so if this element was present in its entirety within the *orf1/orf2* region of these transposons, it would be expected to introduce an additional *Cla*I cut site and thus yield an additional fragment following *Cla*I restriction.

### 5.7 Screening of the *vanX* gene for a point mutation at position 8234

A point mutation at nucleotide position 8234 (G→T) within the *vanX* gene has been associated primarily with porcine isolates of VanA GRE, being relatively uncommon amongst VanA GRE isolated from humans and poultry (Jensen *et al.*, 1998).

The 48 nosocomial GRE isolates were screened for the presence of the point mutation by amplification of a 424-bp fragment internal to the *vanX* gene and subsequent *Dde*I restriction analysis. *Dde*I restriction of the prototype amplicon is expected to give rise to three fragments, whilst the point mutation at nucleotide position 8234 eliminates a *Dde*I cut site, thus resulting in only two fragments.

All 48 isolates yielded three fragments upon *Dde*I restriction, consistent with none of the isolates harbouring the point mutation.

## 5.8 PCR analysis of the Tn*1546* elements of the two general practice VanA GRE

The two VanA isolates identified during the course of the five-month survey of enterococcal epidemiology were subjected to limited PCR analysis of their VanA transposons. The *orf2-vanR* and the *vanX-vanY* intergenic regions were examined by PCR, as described in sections 5.2 and 5.3.

One of the general practice isolates yielded prototype amplicons from the two regions studied, whilst the other gave rise to amplicons consistent with the presence of IS*1542* and IS*1216V*-like elements within the *orf2-vanR* and *vanX-vanY* regions respectively. Restriction analysis confirmed these insertions, with the *vanX-vanY* restriction consistent with a deletion adjacent to the point of IS*1216V*-like insertion. This suggested that the transposon types prevalent amongst nosocomial VanA isolates in Edinburgh can also be found in community isolates.

# Chapter 6: Results

## The impact of Tn*1546* variation on glycopeptide resistance

Large-scale alterations within the *orf1/orf2* region of Tn*1546*-related elements will clearly disrupt the transposase and resolvase functions, which together facilitate the transposition of the transposon. The impact of variation elsewhere within the transposon is, however, less clear. Whilst insertions and/or deletions within genes will clearly disrupt the function of that gene, such variation within intergenic regions may or may not affect the expression of the downstream genes. The resistance phenotypes expressed by the VanA GRE, with and without the Tn*1546* insertions described in the previous chapter, were compared to assess any impact of Tn*1546* variation.

## 6.1 Correlation between Tn*1546* genotype and teicoplanin susceptibility

Those isolates harbouring Tn*1546*-related elements with IS*1542* and IS*1216V*-like insertions upstream of *vanR* and *vanY* respectively were found to have increased levels of teicoplanin resistance when compared with those isolates harbouring prototype Tn*1546* elements. Vancomycin resistance levels were unaltered. Teicoplanin resistance levels conferred by non-prototype elements were clearly greater than those conferred by prototype elements ($P<0.001$) when susceptibility tests were performed on brain-heart infusion media. The same phenomenon was apparent when susceptibility tests were performed on Iso-Sensitest agar ($P<0.001$). The difference between prototype and non-prototype elements was less apparent on Mueller-Hinton agar ($0.05<P<0.1$). The MIC results are summarized in table 6.1. Appendix D shows results of susceptibility testing on the three different media of isolates harbouring prototype and non-prototype Tn*1546* elements.

The fact that the prototype Tn*1546* elements conferred comparable levels of teicoplanin resistance when tested on the three different media indicated that the elevated resistance seen in the non-prototype VanA isolates was not solely due to the differences in the media used (table 6.1).

**Table 6.1: Summary of teicoplanin MIC data for VanA isolates with prototype and non-prototype Tn*1546* elements**

| | Prototype Tn*1546* (n=15) | | | Non-prototype Tn*1546* [a] (n=33) | | |
|---|---|---|---|---|---|---|
| **Media** | **$MIC_{50}$** | **$MIC_{90}$** | **Range** | **$MIC_{50}$** | **$MIC_{90}$** | **Range** |
| ISTA | 8 | 32 | 4 - 32 | 64 | 128 | 16 - 128 |
| MHA | 16 | 64 | 4 - 64 | 32 | 64 | 16 - 128 |
| BHIA | 16 | 32 | 8 - 32 | 256 | 512 | 32 - 1024 |

$MIC_{50}$, $MIC_{90}$ and MIC range expressed in mg/L. [a] Non-prototype Tn*1546* elements defined as having IS*1542* and IS*1216V*-like insertions within the *orf2-vanR* and *vanX-vanY* intergenic regions respectively.

The basis for glycopeptide resistance is the substitution of D-alanine for D-lactate, which is synthesized from pyruvate. It was considered possible, therefore, that if BHI media was significantly richer in either pyruvate or lactic acid than the other two media used in the sensitivity testing, it may explain the results shown in table 6.1. Sensitivity testing was therefore repeated on Isosensitest agar supplemented with varying levels of either pyruvate (sodium pyruvate) or lactate (D,L-lactic acid). The addition of neither component made any impact on the level of teicoplanin resistance. A summary of these results is shown in appendix E.

## 6.2 Identification of a hybrid promoter formed by IS*1542* insertion

Attention turned to the two IS elements present within the non-prototype Tn*1546*-related elements in an attempt to identify features that may influence the expression of the *van* genes.

The IS*1216V*-like insertion within the *vanX-vanY* intergenic region was initially thought to be the most likely cause for the elevation in teicoplanin resistance. Teicoplanin resistance is known to be conferred by the *vanZ* gene, and so if the IS*1216V*-like insertion introduced any feature that may enhance expression of the downstream genes, it would have a direct effect on *vanZ.* Examination of the sequence of the modified *vanX-vanY* region did not reveal any feature likely to enhance gene expression. Indeed, given that the *vanY* and *vanZ* genes are most likely co-transcribed with *vanHAX,* it is more likely that the presence of the IS*1216V*-like insertion within the intergenic region would hinder, rather than enhance, expression of the downstream genes.

However, similar examination of the IS*1542* insertion region identified a hybrid promoter that was formed by the insertion of IS*1542.* The -10 TATAAT box that forms part of the native *vanR* promoter (nucleotides 3926-3931) was duplicated as part of the 8-bp target site duplication generated by IS*1542* insertion at nucleotide position 3932. This duplicated -10 box formed a promoter sequence in conjunction with an outwardly-directed -35 box, which is present within the inverted repeat of IS*1542.* The hybrid promoter sequence is illustrated in figure 6.1.

**Figure 6.1: The native *vanR* promoter, and the hybrid promoter formed by IS*1542* insertion**

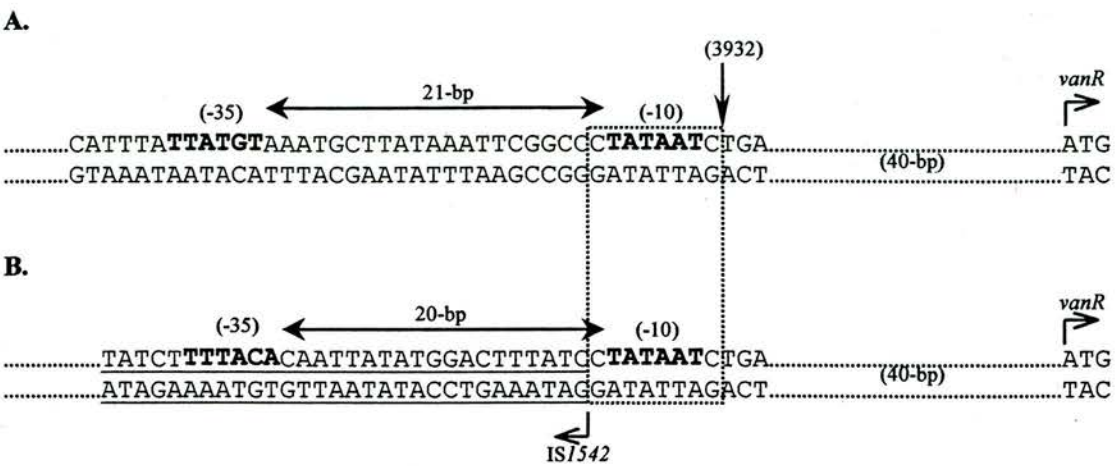



**Figure 3.** Sequences of the proposed *vanR* promoter regions. **A.** *vanR* promoter region present in the prototype *orf2-vanR* sequence, as proposed by Holman *et al.*(1994). **B.** The hybrid *vanR* promoter generated by IS*1542* insertion at nucleotide 3932. The -10 and -35 regions are labelled and highlighted in bold, with the distance between these regions also indicated. The 8-bp target site duplication generated by IS*1542* insertion is indicated by the broken box spanning figures A and B. The start of the *vanR* gene is indicated (ATG; nucleotides 3976-3978), as is the start of IS*1542* (reverse orientation relative to *vanR*) in figure B, with the IS*1542* DNA underlined.

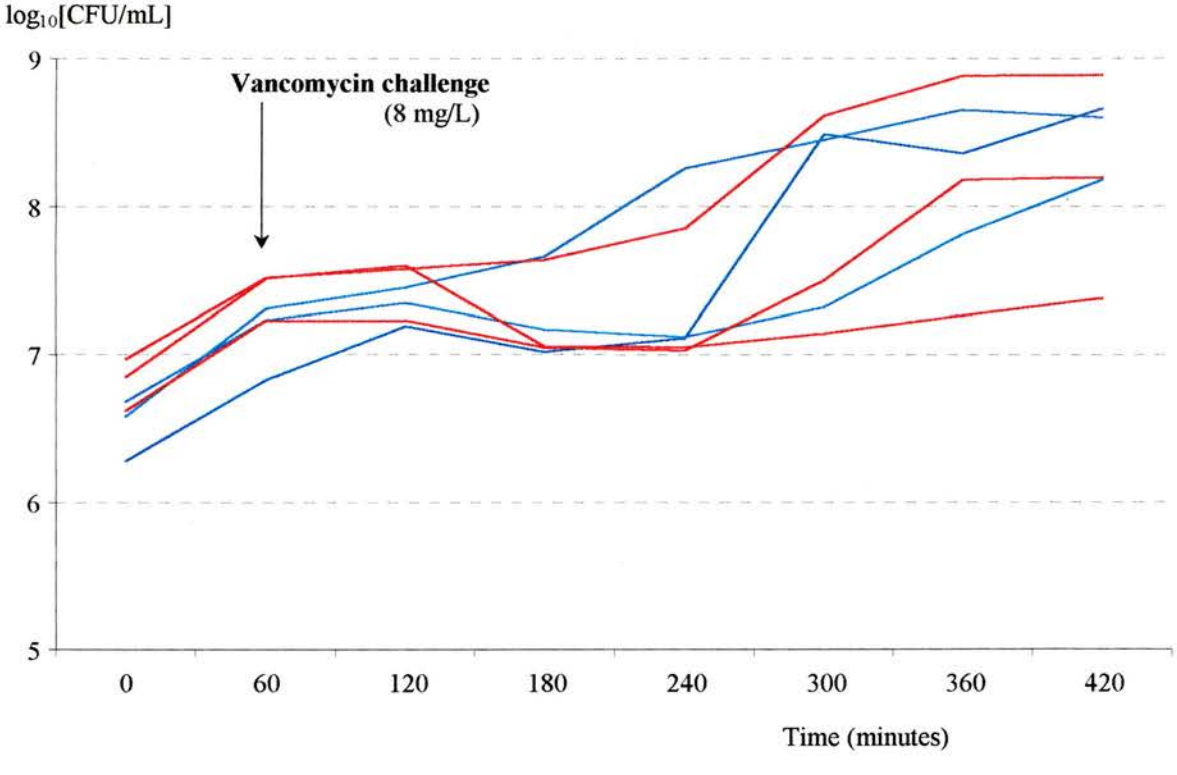
## 6.3 Growth curve analysis of VanA GRE following glycopeptide challenge

The insertion of IS*1542* introduces over 1.3-kb of additional DNA between the native *vanR* promoter and the start of the *vanR* gene. As the *vanRS* genes encode the two-component regulatory system that is ordinarily responsible for the expression of the downstream resistance genes in response to glycopeptide challenge, it was thought possible that the additional DNA may interfere with this inducible resistance gene expression mediated by VanR and VanS. Growth curve analysis was thus performed to determine if glycopeptide resistance was still induced by glycopeptide

challenge, or if it was expressed constitutively, perhaps from the hybrid promoter described in section 6.2.

Figure 6.2 shows the results of growth curve analysis that was performed on a variety of VanA GRE, some harbouring prototype Tn*1546* elements, and some harbouring non-prototype elements containing the IS*1542* insertion.

**Figure 6.2: Growth curve analysis of VanA GRE in response to glycopeptide challenge**




Each line represents a different VanA isolate. Those isolates depicted in blue have non-prototype Tn*1546* elements, harbouring the IS*1542* insertion. Those depicted in red have prototype Tn*1546* elements. Vancomycin was added to cultures to a final concentration of 8mg/L after 60 minutes.

Growth curve analysis showed that the growth rate of all isolates at least slowed upon glycopeptide challenge and, in some cases, growth stopped completely. Generally, growth resumed two to three hours after glycopeptide challenge. This trend is consistent with inducible glycopeptide resistance, for if expression of resistance was constitutive, the rate of growth would be expected to be maintained upon glycopeptide challenge. As shown in figure 6.2, there were no consistent differences in the rate of response to glycopeptide challenge between those isolates with prototype Tn*1546* elements, and those with non-prototype elements.

## 6.4 D,D-dipeptidase activities of prototype and non-prototype Tn*1546* elements

As a measure of *van* resistance gene expression, VanX and VanY enzyme assays were performed on a selection of VanA isolates with and without exposure to glycopeptides. Isolates were selected so as to include those with prototype Tn*1546* elements and those with non-prototype elements that include the hybrid promoter formed by IS*1542* insertion. VanY enzyme assays were unsuccessful, failing to demonstrate any VanY activity in isolates harbouring either prototype or non-prototype Tn*1546* elements. The reason for this is unknown. The VanX assay was, however, successful and the results, expressed as nmoles of D-Ala formed per minute per milligram protein, are shown in table 6.2.

VanX activity conferred by prototype and non-prototype Tn*1546* elements was comparable when expression was induced by glycopeptide exposure (table 6.2). However, discounting isolate RIE-29, VanX activity conferred by non-prototype Tn*1546* elements without induction was on average 10-fold greater than that conferred by prototype Tn*1546* elements in the absence of induction (table 6.2). It

was thought likely that this moderate-level constitutive expression of *vanX* in non-prototype Tn*1546* elements was attributable to the hybrid promoter formed by IS*1542* insertion.

**Table 6.2: VanX activity conferred by prototype and non-prototyype Tn*1546* elements with and without induction**

| Isolate | VanX D,D-dipeptidase specific activity [a] | |
|---|---|---|
| | With induction | Without induction |
| **Prototype Tn*1546*** | | |
| ECH-01 | 176.1 | 5.9 |
| ARI-01 | 245.2 | 4.8 |
| NIN-01 | 227.8 | 3.1 |
| NIN-02 | 214.2 | 1.3 |
| GWI-03 | 193.4 | 2.5 |
| GWI-05 | 209.7 | 2.8 |
| **Non-prototype Tn*1546*** | | |
| RIE-01 | 272.7 | 58.1 |
| RIE-05 | 285.8 | 45.3 |
| RIE-08 | 249.4 | 42.1 |
| RIE-19 | 258.4 | 28.1 |
| RIE-27 | 357.1 | 91.2 |
| RIE-29 | 290.1 | 8.2 |

[a] VanX activities are expressed as nanomoles of D-Ala formed per minute, per milligram protein (nmol $min^{-1}$ $mg^{-1}$). Non-prototype Tn*1546* elements are defined as those harbouring the hybrid promoter generated by IS*1542* insertion within the *orf2-vanR* intergenic region.

The VanX assay clearly demonstrated that the normal induction system was still active, and thus presumably expression from the native *vanR* promoter was unaffected by the IS*1542* insertion. The moderate-level constitutive expression of the *van* genes from the hybrid promoter seemed to supplement, rather than replace, the expression from the native *vanR* promoter.

A notable exception to the trend discussed above was seen in isolate RIE-29 which, despite harbouring a non-prototype Tn*1546* element with the IS*1542* insertion, had a VanX activity only marginally greater than that of isolates harbouring prototype Tn*1546* elements. The *orf2-vanR* intergenic region of isolate RIE-29 was sequenced and the presence of the hybrid promoter was confirmed, with no sequence variation from that shown in figure 6.1. The reason for the lower than anticipated VanX activity in the absence of induction is unclear.

It is interesting to note that isolate RIE-19, used in the VanX dipeptidase enzyme assays, was one of the isolates that was identified by PCR as harbouring both the *vanA* and *vanB* genes. Assuming that the *vanB* gene was indicative of an intact *vanB* gene cluster within RIE-19, it would be anticipated that vancomycin challenge would induce both the VanX and $VanX_B$ enzymes. This would be expected to result in RIE-19 exhibiting higher levels of VanX dipeptidase activity than those isolates harbouring the *vanA* gene cluster alone. As shown in table 6.2, this is not the case. The reason for this is unclear.

# Chapter 7: Discussion

During the last decade, GRE have emerged as a significant cause of nosocomial infection and colonization in both Europe and the USA, having previously been regarded as a low-grade pathogen. The propensity of enterococci, and in particular *E. faecium*, to acquire antibiotic resistance determinants has undoubtedly been a major factor in their emergence as nosocomial pathogens. Whilst in the USA GRE are solely a nosocomial pathogen, their epidemiology in Europe is complex, with GRE readily isolated from a variety of non-hospital sources. A multifaceted approach is required in any attempt to gain an understanding of their epidemiology.

The interest in the epidemiology of GRE was heightened by the controversy surrounding the use, and eventual withdrawal, of avoparcin as a growth-promoting agent in animal livestock production. This controversy and the potential implications for both the animal husbandry and pharmaceutical industries following avoparcin's withdrawal, have resulted in many studies addressing the degree of relatedness of VanA GRE from human and non-human sources.

The application of conventional techniques, such as ribotyping and PFGE, in the study of GRE typically reveals a very heterogeneous population within hospitals, unless isolates are associated with a defined outbreak. With such diversity amongst GRE from human sources, conclusive evidence for strains that are common to man and other sources (particularly animals) is scarce. The diversity of GRE isolates points to the dissemination of VanA resistance by the transfer of plasmids and transposons. Thus interest has turned to investigating the diversity of the Tn*1546* elements themselves.

In the course of this study, 55 isolates of GRE were collected from eight hospitals across Scotland. To gain an understanding of their epidemiology, and to enable comparison of the VanA elements prevalent in Scotland, the 55 isolates were studied by PFGE and all VanA isolates were examined for diversity within their Tn*1546*-related

elements. In addition, all VanA isolates were examined for their ability to transfer glycopeptide resistance, and attempts were made to ascertain the nature of transferable elements mediating glycopeptide resistance gene transfer.

### 7.1 The composition of the GRE isolates studied

The composition of the 55 GRE isolates was consistent with previous published findings. Whilst the majority of cases of enterococcal infection are caused by *E. faecalis*, *E. faecium* is the predominant species in collections of multi-drug resistant enterococci. Thus a collection of enterococcal isolates gathered on the premise that they are glycopeptide-resistant will not reflect the true prevalence of *E. faecalis* against *E. faecium*. For example, of 105 clinical GRE collected from 31 hospitals in the United States, 78% were identified as *E. faecium* (Clark *et al.*, 1993). Similarly, of 116 GRE isolated from six US hospitals, 92% were *E. faecium* (Bopp *et al.*, 1999). In this study 64% of GRE isolates were *E. faecium*, with the remainder being *E. faecalis*. This was in contrast to the collection of glycopeptide-sensitive enterococci (GSE) that was established during the five-month survey of enterococcal epidemiology. Seventy-five percent of the GSE isolates were *E. faecalis*. The predominance of *E. faecalis* in cases of enterococcal infection may reflect the fact that *E. faecalis* is inherently more virulent than *E. faecium*. However, potential virulence determinants within *E. faecium* are currently the focus of much attention and it is likely that such virulence factors will be identified. It is also likely that the multi-drug resistant nature of *E. faecium* becomes a virulence factor, particularly in infections of immuno-compromised patients undergoing aggressive antibacterial therapy.

All isolates were identified to the species level by the API20 Strep system of biochemical tests. All nosocomial GRE isolates were identified as either *E. faecium* or

*E. faecalis*, with the identification showing strong certainty on the basis of the API profile. Whilst the majority of GSE isolates were also identified with certainty as either *E. faecium* or *E. faecalis* by the API20 Strep system, five isolates were misidentified as *E. casseliflavus*. The five isolates failed to yield the *vanC2/3*-specific PCR products that would confirm the API identification. Subsequent PFGE analysis of the isolates suggested one of the five to be an *E. faecium*, with another an *E. faecalis*. The problems associated with the identification of non-classical enterococcal species are well documented (Devriese *et al.*, 1993). Commercially available kits, including the API20 Strep system, are recognized as being unreliable in the identification of enterococcal species other than *E. faecalis* (Miranda *et al.*, 1991; Morrison *et al.*, 1997). Whilst the misidentification of an *E. faecium* isolate may not, therefore, be regarded as unusual, the API20 Strep system would be expected to correctly speciate an *E. faecalis* isolate. If, as suggested, at least one of the misidentified isolates was an *E. faecalis*, it may reflect errors in either the set-up or the interpretation of the API strip. However, it may be significant that the five misidentified isolates were not clinical isolates, but were obtained from the screening of faecal samples. It is likely that the identity of the five isolates could have been resolved by PCR analysis with the species-specific primers described by Dutka-Malen *et al.* (1995).

The proportion of VanA and VanB isolates within the 55 GRE studied was in accordance with published findings. Typically, the VanA phenotype is the most commonly encountered glycopeptide resistance phenotype (Morrison *et al.*, 1997; Bopp *et al.*, 1999). In this study, 78% of the GRE isolates were *vanA* genotype, 13% were *vanB* genotype, and the remaining isolates were found to harbour both the *vanA* and *vanB* genes. Enterococcal isolates in which the *vanA* and *vanB* gene clusters coexist have only rarely been described (Woodford *et al.*, 1997b). It should be noted that there

are exceptions to the 'rule' of VanA predominance. In some hospitals, studies have identified the predominant resistance phenotype to be VanB (Taylor *et al.*, 1999; Nelson *et al.*, 2000). This includes Glasgow's Western Infirmary, from which 11 VanA GRE were collected in the course of this study (Nelson *et al.*, 2000). Such findings highlight the fact that, whilst interhospital spread of GRE certainly occurs, GRE appear to have emerged independently at different hospitals and thus hospitals can have quite distinct GRE populations and epidemiology.

The levels of vancomycin and teicoplanin resistance displayed by VanA and VanB phenotype enterococci were generally consistent with the typical range of sensitivities that would be expected. The one exception to this was a VanB isolate that displayed teicoplanin resistance (MIC 32mg/L). The teicoplanin resistance of this isolate (RIE-28) was not investigated further, although it is likely to have arisen through the constitutive expression of the *vanB* gene cluster, as described by Hayden *et al.* (1993). Teicoplanin sensitivity of VanB enterococci is caused by the failure of teicoplanin to induce *vanB* gene expression (Evers & Courvalin, 1996). Constitutive expression of the genes renders VanB strains resistant to teicoplanin.

Avoparcin MICs were ascertained for many of the VanA and VanB isolates. The results clearly demonstrate the cross-resistance between the glycopeptide agents. More notable, however, is that the VanB isolates display avoparcin resistance, albeit at variable levels. Thus whilst the VanB resistance mechanism is not induced by teicoplanin, it is induced by avoparcin. The apparent lack of VanB GRE in animals and environmental sources does not, therefore, stem from the failure of avoparcin to select for VanB enterococci.

The results of sensitivity testing of non-glycopeptide antibiotics on the VanA GRE, performed primarily to identify co-transferred resistance determinants, were generally

consistent with anticipated results. Once again, however, it should be noted that the collection of GRE isolates does not reflect a typical collection of enterococcal isolates. Isolates collected solely on the basis of their glycopeptide resistance are more likely to be multi-drug resistant than would otherwise be the case if the enterococcal collection were not specifically glycopeptide-resistant. This is illustrated by comparing the studies described by Klare *et al.* (1999) and Leach *et al.* (2000), which looked at antibiotic susceptibilities of a collection of enterococcal isolates and a collection of GRE isolates respectively. Resistance rates were significantly higher amongst the GRE isolates. In general, the resistance rates described in this study were consistent with what would be expected of a collection of GRE isolates, although rates were lower than those described by Leach *et al.* (2000). The isolates studied by Leach and colleagues were, however, solely vancomycin-resistant *E. faecium*, whereas the GRE collection in this study included both *E. faecium* and *E. faecalis*.

In this study, as anticipated, ampicillin resistance was significantly more common in *E. faecium* than in *E. faecalis*. Conversely, tetracycline sensitivity tests performed on the VanA isolates showed a significantly higher prevalence of tetracycline resistance in *E. faecalis* isolates than in *E. faecium*. High-level resistance to streptomycin was detected in 58% of the GRE isolates, being more common amongst *E. faecium* isolates than *E. faecalis* (69% versus 50%). Only five of the 48 isolates displayed HLGR, with four of the five also displaying high-level resistance to streptomycin. Although several new aminoglycoside phosphotransferase enzymes conferring gentamicin resistance have been identified (Chow, 2000), HLGR is most frequently associated with the production of a bifunctional enzyme that confers resistance to all clinically-available aminoglycosides, with the exception of streptomycin (Ferretti *et al.*, 1986). However, the streptomycin adenyltransferase enzyme is often produced by HLGR strains in

addition to the bifunctional enzyme that confers gentamicin resistance, thus rendering the strain resistant to all aminoglycosides (Gray & Pedler, 1992). The four isolates that display high-level resistance to both gentamicin and streptomycin may possess this combination of aminoglycoside-modifying enzymes.

The five-month survey of enterococcal epidemiology was performed primarily to ascertain the degree of diversity amongst clinical enterococcal isolates that did not necessarily display glycopeptide resistance, and to look for any relationships between GRE and GSE. The two isolates that were identified as VanA GRE were both from general practice specimens - no GRE were detected through the routine screening of faecal samples. It should be noted, however, that the method used for the selection of enterococci from faecal samples was not selective for GRE, and thus the fact that no GRE were detected does not mean that none were present. The media used for the routine screening of faecal samples was a modified version of that used by Nelson *et al.* (2000) for the selection of GRE. Vancomycin was omitted from the media, as the primary objective was the isolation of enterococci and not specifically GRE. The likelihood that GRE would go undetected was further increased by the fact that only one suspected enterococcal colony was picked for further study from each faecal sample. In this way, the epidemiology survey was looking at a cross-section of the enterococcal population, rather than being a comprehensive study. It certainly cannot be used to draw any conclusions on the carriage rate of GRE, not least because the number of faecal samples screened was low.

### 7.2 PFGE analysis and interpretation

All isolates collected in the course of this study were analysed by PFGE to assess the degree of clonality within the GRE population and the sensitive enterococcal isolates.

Interpretation of the PFGE patterns was performed according to the criteria described by Tenover *et al.* (1995). These criteria are intended for use in clinical hospital laboratories when examining relatively small sets of isolates (typically no more than 30) which are related to putative outbreaks of disease. Accordingly, although perfectly suited to the investigation of the renal unit outbreak within the Royal Infirmary of Edinburgh, the criteria are less-well suited for interpreting the PFGE patterns of the 94 GSE isolates collected during the five-month epidemiology study.

The interpretation criteria are based on the number of fragment differences that would be expected within PFGE patterns that had undergone a defined number of "genetic events". These genetic events are defined as point mutations that result in the creation, or loss, of restriction sites, and insertions or deletions of DNA. PFGE patterns that differ by two or three fragments are deemed to be closely related on the basis that the fragment differences have arisen through a single genetic event. Two genetic events, according to Tenover *et al.*, would be expected to result in four to six fragment differences and would be interpreted as being possibly related. PFGE patterns with fewer than 50% of fragments in common are considered unrelated, indicative of three or more genetic events that result in seven or more fragment differences. Tenover *et al.* propose that these criteria are reliable if PFGE resolves at least ten distinct fragments. The basis of the criteria is a simplified account of variation within the bacterial genome. Whilst the rationale behind the number of fragment differences that can be expected following a defined number of genetic events appears sound, there is considerable debate over the number of fragment differences that can be expected from clonal isolates.

A point mutation that leads to the creation of an additional restriction site would, according to Tenover *et al.*, result in a three-fragment difference, as might insertion of a

large mobile genetic element (200-300 kb) into the chromosome. In both cases, the resulting PFGE patterns would be classed as closely related to the original pattern, although the two resulting strains differ significantly in their degree of relatedness to the original strain. In addition, transfer of vancomycin resistance associated with large chromosomal elements can markedly alter the biochemical properties of the recipient strain (McAshan *et al.*, 1999). Thus while the number of fragment differences may be small, phenotypic properties of a strain can alter significantly. Whilst many studies report that phenotypic characteristics are not a reliable indicator of clonality (Miranda *et al.*, 1991), such conclusions are based on the assumption that PFGE analysis and, more importantly, interpretation of the PFGE patterns, is a reliable indicator of clonality.

Whilst doubts exist over the relatedness of isolates that differ by up to three fragments, one study has suggested that isolates of a single strain can differ by up to seven fragments (Morrison *et al.*, 1999). Morrison *et al.* analysed 30 isolates of vancomycin-resistant *E. faecium* by PFGE, ribotyping, plasmid profile analysis, biotyping, pyrolysis mass spectrometry (PyMS) and antibiogram analysis. Despite PFGE analysis showing one cluster of isolates to differ by up to seven fragments, the isolates were grouped together by ribotyping, plasmid profile analysis, biotyping and PyMS. These results, combined with the geographical and temporal association of the isolates, led Morrison *et al.* to conclude that these isolates represented a single strain. Thus whilst PFGE is considered the gold standard for bacterial typing, the issue of PFGE interpretation is very much unresolved. High degrees of polymorphism within the PFGE banding patterns of vancomycin-resistant *E. faecium* strains has been described (Morrison *et al.*, 1997), suggesting that genetic events occur frequently in this species, or in certain strains of the species. It is recognized that different bacterial

species vary in the degree of polymorphism that they exhibit (Struelens *et al.*, 1996). It is likely, therefore, that even if criteria such as those defined by Tenover *et al.* (1995) were widely accepted, they could not be universally applied to all bacterial species.

Studies assessing the clonality of GRE isolates by PFGE have, in the past, employed many different criteria in the interpretation of patterns. Whilst many have considered isolates differing by no more than three PFGE fragments to be clonal (Murray *et al.*, 1991; Chadwick *et al.*, 1996; Descheemaeker *et al.*, 1999), other studies have deemed that isolates differing by three PFGE fragments are unrelated (Sader *et al.*, 1994). Given the apparently high degree of genomic polymorphism amongst *E. faecium* isolates (Morrison *et al.*, 1997), it is unsurprising that Sader *et al.* (1994) identified "great genomic variability" when ruling that three fragment differences indicated unrelatedness. The criteria defined by Tenover and colleagues provide some degree of uniformity in the interpretation of PFGE patterns, allowing easier comparison of the conclusions reached by different epidemiology studies. It is then for the individual to decide if they accept the full conclusions drawn from such interpretation, or whether they discount those isolates that differ by 'X' number of fragments. The criteria also have the advantage that they do not require any computer software in their interpretation and thus are easily understandable and accessible. The advantages and disadvantages of computer-aided comparisons of PFGE patterns will be discussed later.

The main problem encountered in the interpretation of PFGE patterns in this study was that the majority of isolates had one or more clusters of low molecular weight DNA fragments (< 100-kb) that were not adequately resolved by the running conditions employed (5-40 second pulse times). To enable these low molecular weight fragments to be resolved, without sacrificing the resolution of the high molecular weight fragments, would have required each isolate to be analysed by PFGE twice, by two

different sets of pulse parameters. Such an approach has been successfully applied by Morrison *et al.* (1999) and Miranda *et al.* (1991). Morrison and colleagues employed linear ramped pulse times of 1 to 10 and 10 to 40 seconds for the separation of fragments below and above 145-kb respectively, with a run time of 40 hours. Miranda *et al.* described good resolution of high molecular weight fragments (200 to 500-kb) with pulse times ranging from 5 to 35 seconds, whilst resolution of low molecular weight fragments was achieved by decreasing the pulse times to a fixed five-second interval (Miranda *et al.*, 1991). Miranda *et al.* also demonstrated that isolates, which had identical high molecular weight fragments, proved to be identical when comparing low molecular weight fragments that were resolved with different running parameters. At the most, analysis of the low molecular weight fragments revealed only one or two fragment differences. Thus if variation in low molecular weight fragments went undetected due to inadequate resolution, the 'worst-case' scenario is that isolates would be classed as identical when, according to the criteria defined by Tenover *et al.* (1995), they ought to be classed as closely related. The inadequate resolution of low molecular weight PFGE fragments in this study is therefore unlikely to have had a significant impact when assessing the clonality of the isolates. In retrospect, however, it would have been beneficial to employ running parameters such as those described by Miranda *et al.* (1991) or Morrison *et al.* (1999) for the PFGE analysis of transconjugants. The potential for variation within low molecular weight fragments to go undetected was clearly demonstrated by the combination of PFGE analysis and *vanA*-probing performed on transconjugant strains. Transconjugants ARB-24 and ARB-26 did not have any apparent fragment differences in their PFGE patterns when compared to the recipient strain, *E. faecalis* JH2-2. Despite this, both transconjugants were identified as harbouring the *vanA* gene within low molecular weight DNA fragments, indicating that

some degree of variation had occurred. The sizes of the fragments that were found to be *vanA*-positive (38 and 53-kb) were within the range of poorly resolved fragments.

Given the temporal association of the isolates originating from the outbreak within the renal unit of the Royal Infirmary of Edinburgh, and the rapid intermixing of patients that occurred within the unit (Brown *et al.*, 1998), it was unsurprising that an outbreak strain was identified. The recognition of an outbreak strain, and the cross-infection that it implies, is a common finding of studies investigating similar clusters of GRE infection (Handwerger *et al.*, 1993; Woodford *et al.*, 1993; Chadwick *et al.*, 1996), although the nature of the outbreak strain varies between studies. Chadwick *et al.* (1996) described an outbreak comprised primarily of VanB *E. faecium*, whilst Woodford *et al.* (1993) described cross-infection with a single strain of VanA *E. faecalis*. In this study, the outbreak strain was a VanA *E. faecium*. Of note was the fact that the outbreak strain that was identified within the renal unit of the Royal Infirmary of Edinburgh was not restricted to that hospital. A further three VanA *E. faecium* isolates were identified that displayed the PFGE pattern of the outbreak strain; two isolates from the Borders General Hospital, Melrose, and one isolate from Edinburgh's Western General Hospital. Such interhospital spread has been described previously, usually occurring between hospitals in close geographic proximity that may share patients and staff (Murray *et al.*, 1991; Sader *et al.*, 1994; Chadwick *et al.*, 1996). It is possible that the movement of patients between hospitals in the Edinburgh/Borders region has facilitated interhospital spread. The movement of hospital staff may also be implicated in the interhospital spread, although no screening of staff was performed during the course of the outbreak. In retrospect, it would have been of interest to compare the PFGE pattern of the outbreak strain (PFGE type A) with representatives of the five epidemic strains of glycopeptide-resistant *E. faecium* identified to date.

Discounting the outbreak strain, PFGE analysis of the 55 nosocomial GRE isolates revealed a largely heterogeneous population, consistent with the well-documented polyclonal nature of GRE. No other isolates from different hospitals shared identical PFGE patterns, although one isolate from the Edinburgh City Hospital and one from Monkland's Hospital, Airdrie, were deemed to be possibly related to two isolates from the Royal Infirmary of Edinburgh. Isolates from different areas of the Royal Infirmary of Edinburgh also showed varying degrees of similarity, from identical to possibly related, suggesting the spread of GRE strains between different wards. Small clusters of related isolates were identified within Glasgow's Western Infirmary and Dundee's Ninewells Hospital. The clusters were distinct from each other and from those PFGE types identified elsewhere. It is not known whether the isolates from Glasgow and Dundee were associated with any identified outbreak, or whether there was any epidemiological link between the sources of the isolates within each hospital.

When studying the data presented in table 3.4, it is notable that there is often variation in the *van* genotype of isolates that belong to the same PFGE type or related subtypes. In one such example, the situation appears to mirror that described by Woodford *et al.* (1997b), where the genotype of an *E. faecium* strain changed from *vanB* to *vanA* through an intermediate isolate that contained both genes on distinct plasmids. In this study, isolates RIE-14 and RIE-17 were closely related to the renal unit outbreak strain (PFGE type A) which was *vanA* genotype. RIE-14, isolated in May 1995, harbours both the *vanA* and *vanB* genes, whereas RIE-17, isolated one month later, contained only *vanB*. It is possible, therefore, that RIE-14 (PFGE type $A_5$) was an intermediate isolate between the outbreak strain (PFGE type A) and isolate RIE-17 (PFGE type $A_7$). The situation is not as 'clear-cut' as that described by Woodford *et al.* (1997b), as the isolates described here were not identical by PFGE. Isolates RIE-18

(*vanA*-positive) and RIE-19 (*vanA*/*vanB*-positive) were, however, identical by PFGE. RIE-19 was isolated one month after RIE-18, and appears to have arisen through the acquisition of *vanB* by a strain identical to RIE-18. These findings clearly indicate that isolates are in an environment containing GRE of different genotypes, and that resistance gene transfer is occurring *in vivo*.

PFGE analysis of the 94 enterococcal isolates collected in the course of the five-month epidemiology survey again identified a largely polyclonal collection of isolates. Whilst ten distinct PFGE types were identified within the 94 isolates, the majority of these types consisted of only two or three isolates. PFGE analysis of this collection of isolates did raise a number of interesting points. Firstly, at least one of the PFGE types (type II) appeared to represent a GSE strain that was endemic within the Royal Infirmary of Edinburgh. All seven isolates belonging to PFGE type II came from the Royal Infirmary, with six of these being isolated from different wards within the hospital. Endemic strains of enterococci have been described previously (Patterson *et al.*, 1991). Secondly, many PFGE types are common to isolates from both hospitalized patients and general practice patients, suggesting that the same enterococcal strains that are present in the community are the cause of infection and colonization within hospitals.

Perhaps the most noticeable result from the PFGE analysis of these GSE isolates is the identification of GSE strains that are closely related to strains of GRE identified some two to three years previously, in the course of the renal unit outbreak. Whilst the two VanA GRE that were isolated from general practice specimens were unique by PFGE, the two predominant PFGE types amongst the GSE isolates (types I and II) were identified as being closely related to PFGE types B and D identified within GRE isolates. Given that the GRE isolates were isolated two to three years prior to the

closely related GSE isolates, and that no related GRE have been isolated since, the most likely explanation for this finding is that some of the GRE isolates responsible for the outbreak in 1995 have since lost their glycopeptide resistance determinant. This may have occurred in response to the decrease in vancomycin usage that was implemented in an attempt to control the GRE outbreak and prevent any subsequent outbreak. Rather than the GRE isolates converting to GSE and then becoming endemic within the hospital, it may be that the same GSE strains were endemic around the time of the outbreak in 1995, and that some organisms acquired, albeit temporarily, glycopeptide resistance. No GSE isolates from around the time of the GRE outbreak were studied by PFGE, so this suggestion cannot be confirmed either way. If these PFGE findings are typical, and reflect the loss of glycopeptide resistance determinants by GRE, they would suggest that the GRE population within the Royal Infirmary of Edinburgh is extremely dynamic.

A consistent finding of the PFGE analysis of both the nosocomial GRE and the GSE isolates is the inability to determine the relatedness of enterococcal isolates on the basis of their phenotypic characteristics. This is highlighted in appendix F, which lists the API profile, antibiotic resistance profile and PFGE type of the 26 isolates that comprised the renal unit outbreak. Within PFGE types, both the API profile and the antibiotic resistance profile is seen to vary. Likewise, isolates with the same API profile were deemed to be unrelated by PFGE analysis. Similar findings have been reported previously (Miranda *et al.*, 1991). Appendix F also lists the Tn*1546* type that was assigned (where applicable) on the basis of the *Cla*I restriction profile. Tn*1546* type is also seen to vary within PFGE types, and isolates unrelated by PFGE are shown to share Tn*1546* types. The issue of Tn*1546* variation is discussed later.

In the latter stages of this study, it was decided to readdress the issue of PFGE interpretation and employ the Bio-Rad Gel Documentation system and Diversity Database software for the comparison of PFGE patterns. The primary objective of this reanalysis of PFGE patterns was to identify the advantages and disadvantages of using such a system for PFGE interpretation, rather than to identify PFGE types that had previously gone undetected, or to discount PFGE types that had been mistakenly identified.

The most striking observation arising from the use of the Diversity Database software was the extreme sensitivity of this type of analysis to any inconsistencies across the gel. Duplicate samples run in the first and last lanes of the same gel will seldom show equal migration rates for the restriction fragments. Owing to time constraints at the time Diversity Database analysis was undertaken, the PFGE gels were not rerun. Instead of the gel image being digitized directly from the gel, images were digitized from the original Polaroid photographs that were used for the original visual comparison of PFGE patterns. These gels were only run with one molecular weight ladder and, as such, proved poor subjects for Diversity Database analysis. Isolates that were deemed to be identical by visual comparison of PFGE patterns had widely ranging percentage similarities according to Diversity Database analysis.

For this type of computer-aided analysis to be successful, the PFGE gels themselves must be extremely clear and should contain multiple molecular weight ladders so as to compensate for variable migration rates across the width of the gel. Another problem that was encountered with Diversity Database analysis was that, following the detection of the bands by the computer, it was always necessary to delete bands that had been wrongly detected due to high background, add bands that had not been detected, and, in some cases, alter the precise location of the detected bands. It was often necessary to

add bands that were part of a cluster of two or three bands that, whilst visible by eye, had only been detected as a single, or perhaps a double band by the computer. Whilst this problem was exacerbated by digitizing a Polaroid image that was two to three times smaller than the actual gel, the same problem was encountered when analysis was performed from PFGE gels themselves. The gel depicted in figure 3.4 was run late in the study and was digitized so as to assess the reliability of band detection from an actual gel. Several bands that were visible by eye were not resolved by computerized detection of the bands. The adjustments that were therefore required following band detection mean that the visual component of PFGE analysis cannot be removed entirely, and thus any bias due to the user's own visual interpretation cannot be avoided.

It is unclear whether other researchers have experienced difficulty in the computer analysis of PFGE patterns. Descheemaeker *et al.* (1999) analysed 132 glycopeptide-resistant *E. faecium* isolates by PFGE, and performed both visual and computerized (GelCompar software, Applied Maths) comparison of the PFGE patterns. Whilst Descheemaeker and colleagues make no mention of any difficulties encountered with GelCompar analysis, they state that "visual and computerized analysis of all *Sma*I patterns obtained revealed 81 clones when three or fewer band differences was used as the criterion to define a different clone". The criteria defined by Tenover and colleagues (1995) were cited, and it thus appears the interpretation of the PFGE patterns was primarily performed by visual comparison, and not using the GelCompar software.

Despite the problems encountered with the computer-aided comparison of PFGE patterns in this study, it is likely to be a useful tool for PFGE analysis, providing that the running conditions of gels, and the layout of samples within gels, are optimized.

Assuming that such optimization successfully overcomes the problems discussed above, Diversity Database analysis would be expected to be far superior to visual comparison and interpretation of large sets of isolates. In this study, the visual comparison of patterns and interpretation according to the criteria described by Tenover and colleagues was certainly adequate for the analysis of the nosocomial GRE isolates from the renal unit outbreak in 1995. However, it is thought that the five-month survey of enterococcal epidemiology would have benefited from the use of the Diversity Database software for the comparison of the PFGE patterns.

### 7.3 Transferability of VanA glycopeptide resistance

Thirty-eight of the 48 *vanA*-positive nosocomial GRE in this study successfully transferred glycopeptide resistance to a sensitive recipient of the same species. The transfer frequency was not determined. Although altering the conjugation conditions may have had an impact on the transferability of glycopeptide resistance, the potential for transfer from the ten remaining isolates was not investigated further.

Resistance determinants identified as having been co-transferred with glycopeptide resistance include high-level gentamicin resistance and resistance to $MLS_B$ antibiotics (Leclercq *et al.*, 1989; Heaton & Handwerger, 1995; Woodford *et al.*, 1995a). In this study, the most frequently co-transferred resistance traits were erythromycin resistance and tetracycline resistance, with chloramphenicol resistance and high-level resistance to streptomycin and gentamicin rarely being co-transferred. It should be noted, however, that no attempts were made to ascertain whether co-transferred resistance determinants identified in this study were genetically linked to the *van* gene cluster. Co-transferred determinants may have been located on different plasmids or mobile chromosomal elements. No ampicillin resistance transfer was detected, suggesting that none of the

isolates studied harboured transferable resistance to β-lactams, mediated either by β-lactamases or PBP5. The gene encoding PBP5 has been identified as being linked to transferable vancomycin resistance in some VanB enterococci (Carias *et al.*, 1998). As discussed in the introduction, β-lactamase-producing enterococci are often no more resistant at the inoculum used for sensitivity testing than non-β-lactamase-producers, and thus may go undetected (Murray, 1992). However, the *E. faecium* GE-1 strain used as recipient in conjugation experiments is hypersusceptible to β-lactam antibiotics (Eliopoulos *et al.*, 1982). Therefore, if β-lactamase-mediated resistance had been transferred, it is anticipated that it would have been detectable as an increase in ampicillin resistance. The co-transfer of resistance determinants occurred more frequently from *E. faecalis* than from *E. faecium*. The apparent absence of any co-transferred determinants from the majority of *E. faecium* isolates may reflect the fact that many appear to share the same transferable genetic element, as concluded from the results of PFGE analysis and *vanA* hybridization studies. The exact nature of this transferable element, discussed below, could not be ascertained.

Despite the ready transfer of glycopeptide resistance from the majority of VanA GRE isolates, no *vanA*-positive plasmid DNA was extracted from any of the isolates used in plasmid extractions. Many extractions failed to yield any plasmid DNA, with the only band (usually a poorly defined, broad band) equating to approximately 23-kb linear DNA. A similar band was sometimes obtained from plasmid extractions that were performed on plasmid-free recipients, and this suggested that the band was perhaps fragmented chromosomal DNA. Whilst an alternative possibility was that the fragmented DNA was plasmid DNA, the plasmid being too large to be extracted intact by the plasmid extraction protocol employed, the failure to cure strains of plasmid

DNA, and thus glycopeptide resistance, suggested this was not the case. However, the sensitivity of the curing experiments cannot be estimated as viable counts of the exposed cultures were not performed. The failure to cure plasmid DNA may, therefore, reflect low sensitivity rather than the absence of plasmid DNA. Whilst it had been hoped that electrophoresis of 'intact' genomic DNA (unrestricted PFGE plugs) would enable the identification of plasmid DNA, the technique also yielded a common Tn*1546*-positive band of approximately 23-kb linear DNA.

The combination of PFGE analysis and *vanA* hybridization studies of the pulsed-field gels suggested that the majority of VanA *E. faecium* isolates possess a common genetic element that is responsible for the transfer of glycopeptide resistance. The size of this element, based on the size of *vanA*-positive fragments within donors and transconjugants, and the size of the additional DNA fragments seen in transconjugants, was estimated at approximately 160-kb. Despite the inability to extract intact plasmid DNA and the unsuccessful attempts at plasmid curing with ethidium bromide, the combined results of PFGE and *vanA*-probing suggest that this 160-kb element is, indeed, a plasmid. The fact that the size of the *vanA*-positive fragments in donors matched the size of those in transconjugants is inconsistent with a mobile element that is integrated into the chromosome, unless the *vanA* gene cluster was internal to the mobile element, and flanked by *Sma*I restriction sites. When Handwerger and Skoble (1995) identified a chromosomal mobile element carrying the *vanA* gene cluster, the size of *vanA*-positive fragments in transconjugants differed from that seen in the donor strain. The presence of a 160-kb plasmid is further supported by the fact that the majority of *E. faecium* transconjugants have a common additional DNA fragment (115-kb) that is matched by a DNA fragment within the PFGE pattern of the donor strains. Again, this common band size between donors and transconjugants is inconsistent with

the mobile element being integrated into the chromosome. The most likely scenario, therefore, is that of a 160-kb plasmid, which is cut twice by *Sma*I, generating two fragments: a 45-kb fragment containing Tn*1546*, and a 115-kb fragment. It is interesting to note that none of the transconjugants exhibiting the combination of the 45-kb *vanA*-positive fragment and the 115-kb fragment displayed any co-transferred resistance determinants. This suggests that the common mobile element only harbours the *van* gene cluster and no other resistance determinants.

At least two assumptions have been made in the interpretation of the PFGE and *vanA*-probing studies discussed above. Firstly, it was assumed that the 45-kb fragment shown to be *vanA*-positive, and the 115-kb fragment evident in transconjugants, are part of the same mobile genetic element. This assumption was made primarily on the basis of the close association between the two fragments (table 4.2). The second assumption made was that the 115-kb additional fragment seen in the transconjugants is the same fragment as the 'matching' fragment identified in the donor strains. If either of these assumptions were incorrectly made, it would cast doubt on the proposed 160-kb plasmid.

A technique employed by Quintiliani and Courvalin (1994) may have helped to resolve this issue. In the course of identifying *vanB*-containing chromosomal insertions, they employed zero integrated-field electrophoresis (ZIFE). Agarose plugs were prepared as for PFGE analysis and digested overnight with *Sfi*I. This resulted in only six DNA fragments that were clearly resolved by ZIFE. This technique enabled easy identification of any additional DNA that may have integrated into the chromosome, as only six fragments were being compared, as opposed to the 15-20 fragments that are generated by *Sma*I restriction.

Assuming the identification of a 160-kb plasmid is correct, the consistent identification of *vanA* within the 45-kb fragment may be explained by the findings of Tn*1546* analysis that identified substantial alterations within the *orf1*/*orf2* region of the majority of transposons. Such alterations would disrupt the activities of the transposase and/or resolvase enzymes and thus prevent the movement of the Tn*1546*-related element within the genetic element in which it is located.

It seems likely that the apparent lack of success in extracting plasmid DNA was due to the inability to extract the large plasmid intact, rather than the actual absence of any plasmid DNA. The reason for the failed attempts at plasmid curing with ethidium bromide is unknown, as is the reason for the inability to identify plasmid DNA by the electrophoresis of 'intact' genomic DNA. The plasmid extraction technique that was applied in this study has been used to identify gentamicin resistance plasmids in enterococci (Woodford *et al.*, 1993). The plasmids identified ranged in size from 53 to 80-kb. Typically, *vanA* or *vanB*-bearing plasmids range in size from 26 to 60-kb (Leclercq *et al.*, 1988, 1989; Clark *et al.*, 1993; Handwerger *et al.*, 1995; Heaton & Handwerger, 1995; Woodford *et al.*, 1995b). However, Werner *et al.* (1999) used a modified plasmid extraction protocol, based on that described by Woodford *et al.* (1993) and used in this study, to successfully extract large conjugative *vanA* plasmids ranging from 125-kb to 200-kb. The modifications to the protocol included doubling the length of lysozyme treatment, and performing ethanol precipitation of DNA overnight. Gentle handling of samples was employed throughout. Such modifications may have helped in the isolation of the 160-kb plasmid in this study. Assuming the genetic element identified here is a plasmid, it appears to be amongst the largest *vanA*-bearing plasmids identified to date.

## 7.4 Tn*1546* diversity and the impact of variation on glycopeptide resistance

Different studies examining the diversity of Tn*1546*-related elements have, in general, identified similar forms of variation within the VanA elements, although the prevalence of these non-prototype elements varies significantly between studies. For example, Woodford *et al.* (1998) described a very heterogeneous set of Tn*1546*-related elements within 106 VanA GRE studied. Whilst 19% of isolates harboured a prototype Tn*1546* element, the remaining isolates harboured non-prototype elements with various insertions and deletions within the *orf1/orf2* region and the *orf2-vanR* and *vanX-vanY* intergenic regions. Twenty-three distinct types of non-prototype Tn*1546*-related elements were identified. The findings of Willems *et al.* (1999) were in accordance with those of Woodford and colleagues. Willems *et al.* described Tn*1546* diversity amongst 97 VanA GRE. Twenty-one percent of isolates harboured a prototype Tn*1546* element, whilst the remaining isolates harboured various non-prototype elements disrupted by insertions and deletions.

In contrast, Descheemaeker *et al.* (1999) reported a remarkably conserved set of Tn*1546* elements in 132 VanA *E. faecium* studied. Over 80% of the strains studied harboured one of two transposon types, one being the prototype element, and the other differing only in the loss of a *Dde*I restriction site through the previously described point mutation at nucleotide position 8234. Only nine of the 132 isolates were identified as containing Tn*1546*-related elements harbouring IS elements in the *orf1/orf2* region, or within the *orf2-vanR* or *vanX-vanY* intergenic regions.

The results presented in this thesis are generally more consistent with the findings of Woodford *et al.* (1998) and Willems *et al.* (1999) than with Descheemaeker *et al.* (1999). Indeed, the predominant non-prototype Tn*1546* element identified in this study (type B) appears not too dissimilar to the predominant Tn*1546* type (type H) that was

identified by Woodford *et al.* (1998). The two types harbour IS*1542* and IS*1216V*-like insertions within the *orf2-vanR* and *vanX-vanY* intergenic regions respectively. Tn*1546* type H, identified by Woodford *et al.*, was the most common Tn*1546*-related element identified in human enterococcal isolates. It was rarely observed in enterococci from other sources.

Twenty-nine percent of the VanA isolates in this study were identified as harbouring a prototype Tn*1546* element. The remaining isolates harboured non-prototype Tn*1546*-related elements that contained IS*1542* within the *orf2-vanR* intergenic region and at least two copies of an IS*1216V*-like element. Despite the majority of isolates harbouring non-prototype Tn*1546* elements, marked by insertions and deletions, there is a remarkable degree of uniformity in these non-prototype elements. All non-prototype Tn*1546* elements contain both IS*1542* within the *orf2-vanR* intergenic region and the IS*1216V*-like element within the *vanX-vanY* intergenic region. The majority of non-prototype elements are distinguished from one another solely on the basis of variation within the *orf1/orf2* region. In contrast, the non-prototype Tn*1546* elements identified by Woodford *et al.* (1998), and subsequently characterized by Palepou *et al.* (1998), were more heterogeneous in nature. The same insertions were identified within the *orf2-vanR* and *vanX-vanY* intergenic regions, but the insertions occurred separately in some Tn*1546*-related elements, as well as together in other elements.

The IS*1542* and IS*1216V*-like elements have both previously been described at the same nucleotide positions within Tn*1546* as those described here (nucleotides 3932 and 8839 respectively) (Woodford *et al.*, 1998; Descheemaeker *et al.*, 1999; Willems *et al.*, 1999). The fact that the IS*1542* and IS*1216V*-like elements frequently, if not exclusively, occur at the same positions within Tn*1546*-related elements suggests that both elements have some degree of specificity for a target sequence. Specific

consensus target sequences are involved in the transposition of several IS elements, with specificity arising from the recognition of a specific target sequence, a structural or functional feature of the DNA, or a combination of these factors (Galas & Chandler, 1989). IS*1216V* and related elements have been identified at various positions within Tn*1546* (Descheemaeker *et al.*, 1999; Willems *et al.*, 1999), however, comparison of sequences around these published insertion sites failed to identify any specific consensus sequence. This suggests that if specificity does exist for IS*1216V*-like insertion, it is a structural or functional feature of the DNA. No similar comparison of IS*1542* insertion sites was possible, as it has only been identified at nucleotide position 3932.

As witnessed in this study, small-scale deletions have often been identified adjacent to the point of IS*1216V* insertion within the *vanX-vanY* intergenic region (Willems *et al.*, 1999). IS elements are known to induce alterations within the surrounding DNA (Galas & Chandler, 1989). Many IS elements have been identified as inducing deletions, increasing the deletion frequency by 30- to 2000-fold. Such deletions typically terminate precisely at one end of the IS element, as seen in the isolates in this study that harboured an IS*1216V*-like insertion with an adjacent 93-bp deletion within the *vanX-vanY* intergenic region.

It is interesting to speculate that whilst IS*1542* and IS*1216V*-like elements may differ in their abilities to induce deletions, the apparent absence of deletions immediately adjacent to the point of IS*1542* insertion may reflect the nature of the surrounding DNA. IS*1216V*-like elements frequently insert at nucleotide position 8839 within the *vanX-vanY* intergenic region, some 200-bp downstream from the end of the *vanX* gene. Thus sizeable deletions upstream from the point of insertion can take place without affecting glycopeptide resistance. Deletions downstream of the insertion point

would, at most, disrupt the function of the non-essential accessory genes. Conversely, deletions either upstream or downstream from the point of IS*1542* insertion (nucleotide position 3932) would be expected to disrupt the expression of glycopeptide resistance. The insertion site is located just 44-bp upstream from the start of the *vanR* gene, thus a deletion within this region would disrupt the expression of the VanRS two-component regulatory system. Any deletion upstream of the insertion point would remove the native *vanR* promoter that runs from nucleotide 3899 to 3931. The results of the VanX enzyme assays following induction suggest that the native *vanR* promoter is still active despite the insertion of IS*1542*. Thus if the promoter was deleted by an IS*1542*-induced deletion, it is likely that the expression of glycopeptide resistance would at least be decreased, and perhaps lost altogether. If the insertion of IS*1542* does, on occasion, induce deletions, it would therefore be likely that they would go undetected. Such deletions would result in glycopeptide sensitivity, and so there would be no reason to examine for a Tn*1546*-related element.

A prominent feature of the majority of Tn*1546*-related elements identified in this study is substantial rearrangement within the 5' region of the transposon that corresponds to the *orf1/orf2* genes, encoding the transposase and resolvase enzymes. Other studies have reported similar findings, with the variation usually attributable to the insertion of IS*1216V* and/or IS*3*-like elements with accompanying deletions. The combination of IS*1216V* and a truncated IS*3*-like element within the 5' region of Tn*1546* was first identified by Handwerger and Skoble (1995), and appears to arise through sequential insertion of the IS elements. This combination of IS elements, associated with the loss of the first 120-bp of the transposon, has since been identified in other studies (Jensen *et al.*, 1998; Palepou *et al.*, 1998; Willems *et al.*, 1999). The two IS elements have also been identified separately within the *orf1/orf2* region.

Willems *et al.* (1999) describe IS*1216V* in various positions within the *orf1* and *orf2* genes, whilst the most common Tn*1546* type identified by Woodford *et al.* (1998) contained an IS*3*-like/*orf1* complex, but lacked the IS*1216V*/IS*3*-like complex (Palepou *et al.*, 1998). The majority of Tn*1546*-related elements identified in this study as having variation within the 5' region appear to have any insertions and deletions restricted to the first 905-bp of the transposon, and all non-prototype transposons appear to have at least one copy of an IS*1216V*-like element within the 5' region. The IS*3*-like element described above was not screened for.

Five isolates in this study failed to yield an L-PCR amplicon with the Tn*1546*-IR primer alone. Interestingly, these five isolates were clonally related to one another, belonging to PFGE type A and closely related subtypes. The five isolates may have undergone variation within the *orf1* region that has removed the left inverted repeat. The failure of L-PCR, associated with such Tn*1546* alterations, has been described previously (Palepou *et al.*, 1998).

It was proposed that Tn*1546* type D, identified in this study in isolate RIE-11, may harbour an IS element within the *vanS* gene. This proposal was on the basis that the *Cla*I restriction fragment corresponding to the region from IS*1542* (within the *orf2-vanR* intergenic region) to *vanH* is larger than anticipated. The isolate was previously identified as having a prototype *vanS-vanH* intergenic region. Darini *et al.* (1999b) described a VanA *E. faecium* isolate with an IS*1216V* insertion within the 3' end of the *vanS* gene. Whilst IS*1216V* insertion at the position described by Darini *et al.* would most likely have been detected by PCR with the *vanS*-f and *vanH*-r primers used in this study, it is possible that a similar insertion has occurred elsewhere within the *vanS* gene. Disruption of the *vanS* gene does not affect glycopeptide resistance (Arthur *et al.*, 1992c). It is thought likely that VanR-dependent transcription in the absence of

VanS may proceed through cross-talk with other two-component regulatory systems encoded by the host chromosome.

Perhaps the main question arising from the PCR analysis of the Tn*1546*-related elements is why L-PCR with Tn*1546*-IR primer alone and in combination with the *vanR*-r primer, failed to yield any product from non-prototype VanA elements until the latter stages of the study. And why, despite the eventual success of the L-PCR with Tn*1546*-IR primer alone, the results of IR-probing experiments suggested that the majority of Tn*1546*-related elements contain only one Tn*1546*-IR primer-annealing site.

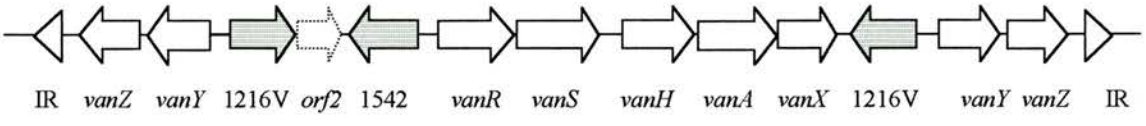
In theory, the failure of L-PCR with the Tn*1546*-IR primer in conjunction with the *vanR*-r primer may be explained by the unmatched nature of the primers. The two primers differ significantly in their length and melting-temperatures. However, the Tn*1546*-IR primer was successfully applied with the *vanX*-f primer to confirm the integrity of the 3' end of the Tn*1546*-related elements. The *vanX*-f and *vanR*-r primers are comparable in their length and melting-temperatures, and so given that L-PCR with the Tn*1546*-IR and *vanX*-f primers was successful, it seems unlikely that the failure of Tn*1546*-IR/*vanR*-r L-PCR can be solely attributable to the use of an unsuitable primer combination.

Whether the paradox of the successful Tn*1546*-IR L-PCR, but only one apparent Tn*1546*-IR annealing site, reflects variation within the left-hand inverted repeat would require sequencing to confirm. Sequencing would need to be performed from a site within the Tn*1546*-related element, going back towards the left-hand inverted repeat. Such an approach is complicated by the fact that the sequence downstream of the inverted repeat is not known, given the uncertain nature of the variation within the *orf1*/*orf2* region. It may be that conditions within the L-PCR reaction were better able

to compensate for degeneracy within the primer-annealing site than the oligonucleotide probing conditions with the Tn*1546*-IR primer. However, initial hybridization conditions in IR-probing experiments were not particularly stringent, and lowering the stringency further did not result in the identification of more IR-positive fragments.

An alternative explanation for the single IR-positive fragment seen in many of the non-prototype Tn*1546* elements is that an inverted duplication of the 3' end of Tn*1546* has replaced part of *orf1*. Darini *et al.* (2000) reported such a phenomenon within the Tn*1546* element of a VanA GRE stored at 4°C for a prolonged period. The genetic organization of the resulting Tn*1546*-related element is shown in figure 7.1.

**Figure 7.1: Genetic organization of a Tn*1546*-related element arising from inverted duplication of the 3' end of Tn*1546***




1216V and 1542 represent the IS*1216V*-like and IS*1542* elements respectively. IR indicates a terminal inverted repeat. The *orf2* gene is truncated due to the inverted duplication.

Figure adapted from Darini *et al.*, 2000

As shown in figure 5.11 (page140), the IS*1216V*-like element contains a single *Cla*I restriction site. Thus, in a situation like that shown in figure 7.1, *Cla*I restriction will yield two identical fragments, one from the 5' end of the transposon and one from the 3' end. Both fragments will be IR-positive (both containing the right-hand inverted repeat) but will appear as a single fragment following gel electrophoresis. Confirmation of such an arrangement could be achieved by *BamH*I restriction of Tn*1546*-related elements and probing for either the *vanY* or *vanZ* genes. The non-

prototype Tn*1546*-related elements identified in this study cannot be exactly like the transposon depicted in figure 7.1, as L-PCR analysis confirmed the integrity of *orf2* and most of *orf1* (from nucleotide position 906) in the majority of isolates. However, the inverted duplication may have replaced a section of *orf1* restricted to the first 905-bp.

An interesting observation from the results of Tn*1546* analysis is that certain types of variation appear restricted in their geographical distribution. The deletion immediately adjacent to the point of insertion of the IS*1216V*-like element within the *vanX-vanY* intergenic region is only seen in VanA GRE from the Edinburgh/Borders region. Of the 28 VanA GRE from the Edinburgh/Borders region that harboured a non-prototype Tn*1546*-related element, 26 had the 93-bp deletion adjacent to the point of insertion. Whilst only five VanA isolates from outwith the Edinburgh/Borders region harboured non-prototype Tn*1546*-related elements, none of these isolates had the deletion adjacent to the point of IS*1216V*-like insertion. This suggests that different areas of Scotland have differing Tn*1546* profiles, although more isolates, particularly from outwith the Edinburgh/Borders region, would need to be studied in order to confirm this.

Comparing the results presented here with those of similar studies highlighted a further possible example of varying geographical distribution of Tn*1546* types. The insertion of IS*1542* within the *orf2-vanR* intergenic region was first described by Woodford *et al.* who identified the element in 37 of the 106 VanA isolates studied (Woodford *et al.*, 1998). Thirty-four of the 50 VanA isolates in this study (including the two VanA general practice isolates) were found to harbour Tn*1546*-related elements with the IS*1542* insertion. In comparison, three similar studies in continental Europe, which have between them looked at over 260 VanA GRE, have only identified one isolate from outwith the UK that harbours what is presumably IS*1542* within the *orf2-*

*vanR* intergenic region (Jensen *et al.*, 1998; Descheemaeker *et al.*, 1999; Willems *et al.*, 1999). Descheemaeker *et al.* (1999) described one isolate of human origin with an "IS*256*-like element at position 3932 in the *orf2-vanR* intergenic region". Limited geographical distribution of certain IS elements within Tn*1546* has been described previously. IS*1251* was identified within the *vanS-vanH* intergenic region of VanA GRE from the United States (Handwerger *et al.*, 1995). Only recently has the same variation within the *vanS-vanH* intergenic region been identified in European isolates (Simonsen *et al.*, 2000), suggesting recent intercontinental spread. How certain IS elements can become established within, and restricted to, a certain geographical area is unclear, but such restricted IS element distribution may be of use as an epidemiological marker.

A major issue concerning the use of Tn*1546* diversity as an epidemiological marker is the potential instability of Tn*1546* variation. Distinct Tn*1546* types may differ by only one insertion event, and the IS elements responsible are frequently found elsewhere within the enterococcal genome. Tn*1546* types can thus be altered by a single transposition event. Tn*1546* instability has been described previously, both within the clinical setting and during prolonged cold storage of the GRE isolates (Woodford *et al.*, 1998; Darini *et al.*, 2000). To examine Tn*1546* stability, Woodford *et al.* studied the VanA elements of 12 isolates of the epidemic strain EVREM-3. Six distinct types of Tn*1546* were recognized within the 12 isolates. In addition, the VanA elements varied within three isolates from various stages of a prolonged outbreak on a single hospital unit, suggesting that the VanA element may have undergone some alteration during the outbreak. The results presented in this thesis suggest similar Tn*1546* instability in clonal isolates. Within PFGE type A (and closely related subtypes), three distinct Tn*1546*-related elements were seen, differing from each other

primarily within the *orf1/orf2* region. In addition, Tn*1546* instability during cold storage of isolates was apparent, being exemplified by isolate RIE-25 (Tn*1546* type E). The nature of the alteration that had occurred was not ascertained, although it appears to have been a structural change of the 3' region, associated with the presence of IS*1216V* within the *vanX-vanY* intergenic region. Some IS elements are known to induce alterations within surrounding DNA, including inversions (Galas & Chandler, 1989). Darini *et al.* (2000) describe an inverted copy of the IS*1216V-vanY-vanZ*-inverted repeat block from the 3' end of a Tn*1546*-related element replacing the first 3417 nucleotides of the transposon, the alteration taking place during cold storage of the isolate.

These findings do cast doubt over the validity of Tn*1546* diversity as an epidemiological marker. However, such analysis is still likely to be of use in localized studies, perhaps in conjunction with other techniques. In this study this is perhaps best illustrated by isolate RIE-30 which, although isolated in the Royal Infirmary of Edinburgh, was thought to have originated in the United States. RIE-30 was isolated in December 1997 from a patient, ordinarily resident in the Edinburgh area, who was transferred to the Royal Infirmary of Edinburgh following intensive care treatment in a hospital in the US. Upon arrival at the Royal Infirmary, the patient was found to be both MRSA and GRE-positive, and it was assumed that both had been acquired during hospital treatment in the United States. However, studies showed that whilst the GRE isolate was unique by PFGE analysis, it harboured a Tn*1546*-related element identical to those predominating in Edinburgh hospitals (Tn*1546* type B). The time-scale of isolation made it unlikely that the GRE isolate was acquired by the patient following transfer to the Royal Infirmary. It seems likely, therefore, that the isolate was not actually acquired whilst in the US, but was of an endogenous origin, having been

acquired previously either from a community source or during a previous hospital stay in the Edinburgh region. Further studies support this hypothesis, suggesting that the 160-kb genetic element that carries Tn*1546* in the majority of nosocomial VanA *E. faecium* in Edinburgh is also present in isolate RIE-30. The possibility that the same Tn*1546*-related elements prevalent amongst nosocomial GRE are also present in the community setting was also suggested by the limited PCR analysis performed on the two VanA GRE isolates from general practice specimens. One of these isolates was found to harbour a Tn*1546*-related element with IS*1542* and IS*1216V*-like insertions.

IS elements can have both positive and negative effects on the expression of downstream genes. In general, IS elements are expected to exert a strong polar effect on the downstream genes, with the simplest explanation being that the inserted elements carry internal transcription terminators (Galas & Chandler, 1989). Thus it could be expected that the expression of the *vanY* and *vanZ* genes, most likely co-transcribed with the *vanHAX* genes situated upstream, would be disrupted by the insertion of IS*1216V*-like elements within the *vanX-vanY* intergenic region. This is inconsistent, however, with the results of this study which showed that the level of teicoplanin resistance is elevated, rather than lowered, in those isolates harbouring non-prototype Tn*1546*-related elements. The VanY enzyme assays were unable to establish whether *vanY* was still expressed. However, Willems *et al.* (1999) describe a deletion within the *vanY* gene that was associated with reduced resistance to teicoplanin. They proposed that the *vanY* deletion affects the transcription of the *vanZ* gene that is known to confer teicoplanin resistance. Therefore, with disruption of the *vanYZ* genes being associated with a decrease in teicoplanin resistance, it seems unlikely that the IS*1216V*-like insertion is exerting any negative effect on the downstream genes.

Activation of downstream genes has been observed for several IS elements, and is generally due either to the presence of outwardly-directed promoters within the element, or to the formation of new promoters (hybrid promoters) upon insertion. The latter scenario is that displayed by IS*1542* insertion within the *orf2-vanR* intergenic region. IS*1542* is just one of many IS elements that have potential outwardly-directed -35 regions within their inverted repeat, and thus have the potential to form hybrid promoters in conjunction with a -10 region situated just downstream of the insertion site (Galas & Chandler, 1989). Transcription of antibiotic resistance genes from IS-generated hybrid promoters has been described previously (Galas & Chandler, 1989), although not within the *van* gene cluster. Whilst the results presented in this thesis are the first description of an IS-generated hybrid promoter leading to expression of the *van* genes, the potential for this to happen was first illustrated by Handwerger *et al.* (1995). They described the IS*1251* element within the *vanS-vanH* intergenic region and identified a perfect -35 promoter consensus region (TTGACA) within the right inverted repeat of the IS element. However, the orientation of the IS element was such that any hybrid promoter would be formed at the 3' end of the *vanS* gene, in the opposite direction to which the genes are transcribed. As the inverted repeats of IS*1251* are imperfect, no -35 region is present in the left inverted repeat that may have had the potential to form a hybrid promoter upstream of *vanH*. Examination of the sequence of the IS*1216V*-like insertion within the *vanX-vanY* intergenic region failed to identify any hybrid promoter that may have enhanced the expression of the *vanY* and *vanZ* genes.

Another possible explanation that was considered for the apparent association between IS elements and an elevated resistance to teicoplanin was that the IS elements (either IS*1542* or the IS*1216V*-like element) harboured a P-VanR binding site that would enhance the expression of the downstream *van* resistance genes. Holman *et al.*

(1994) identified a 12-bp sequence that was present in one copy at the *vanR* promoter and in two copies at the *vanH* promoter. The sequence was located within the P-VanR footprinted regions of the two promoters and was thus proposed as serving as a consensus recognition site for P-VanR binding. Examination of the sequences of the IS*1542* and IS*1216V*-like elements failed to identify any 12-bp sequence displaying homology to that described by Holman *et al.*. Gel mobility shift experiments and DNaseI footprinting would be required to confirm this either way. It is, however, unlikely that if the IS elements did harbour such a P-VanR binding site, that it would have any effect on downstream gene expression, as the P-VanR binding site would probably need to be associated with a promoter sequence.

The results presented in this thesis, therefore, suggest that IS*1542*, and not the IS*1216V*-like element, exerts an effect on the downstream genes, by means of a hybrid promoter that leads to constitutive expression of the downstream genes, albeit at a lower level than that seen following induction. Figure 7.2 compares the sequence of the native *vanR* promoter, the hybrid promoter and the consensus sequence of the *Escherichia coli* $\sigma_{70}$ promoter.

**Figure 7.2: Comparison of the native *vanR*, hybrid, and *Escherichia coli* $\sigma_{70}$ promoter sequences**

| | |
|---|---|
| **Native *vanR* promoter:** | TTATGT.....(21-bp).....TATAAT |
| **Hybrid promoter:** | TTTACA.....(20-bp).....TATAAT |
| ***Escherichia coli* $\sigma_{70}$ promoter:** | TTGACA.....(17-bp).....TATAAT |

The sequence of the hybrid promoter matches that of the *Escherichia coli* $\sigma_{70}$ promoter more closely than the native *vanR* promoter and may, therefore, be considered to be a stronger promoter. However, direct comparison of the two promoters (native *vanR* and hybrid) on the basis of their sequences is not actually possible, due to the influence of P-VanR binding. When the *vanR* promoter was identified, it was initially proposed that P-VanR binding at the promoter had a negative effect, as the binding site overlapped the -35 region of the promoter (Holman *et al.*, 1994). If this were indeed the case, it would have helped to explain the enhanced gene expression that appears to follow IS*1542* insertion. The hybrid promoter would be distanced from the P-VanR binding site by the IS*1542* insertion, and thus be free from any inhibition. However, subsequent studies have shown that P-VanR binding has a positive effect on the *vanR* promoter (Arthur *et al.*, 1997), and that it essentially acts as a catalyst for gene expression from the *vanR* promoter, replacing the -35 region of the promoter. As the -35 region of the native promoter is thus largely obsolete, no conclusions can be drawn on the comparative strengths of the native and hybrid promoters solely on the basis of their sequences, as they differ solely in their -35 region.

It remains to be explained why constitutive expression of the *van* genes from the hybrid promoter formed by IS*1542* insertion manifests itself as elevated teicoplanin resistance, and why this is particularly apparent on rich media such as BHI. The fact that this phenomenon has not been witnessed previously, despite similar studies identifying similar transposon types to those described here, is most likely due to the fact that the elevation in resistance is not prominent when susceptibility tests are performed on standard, NCCLS-approved media such as ISTA or MHA.

Arthur *et al.* (1996a) described a gradual increase in vancomycin resistance levels when the proportion of D-Ala-D-Ala-terminating precursors within the cell wall

decreased from 43% to 2%, but a more complete elimination of such precursors was required in order to achieve high-level teicoplanin resistance. Such differing abilities of the two glycopeptides to act against residual D-Ala-D-Ala-terminating precursors may offer some explanation for the elevation in teicoplanin resistance but not vancomycin resistance. Significant constitutive expression of the *van* genes, mediated by the IS*1542*-generated hybrid promoter in the absence of induction, would be expected to lower the proportion of D-Ala-D-Ala-terminating precursors within the cell wall. With expression from the native *vanR* promoter then adding to that from the hybrid promoter following induction, the removal of D-Ala-D-Ala-terminating precursors may be more rapid and complete, given the already reduced levels present within the cell wall. Analysis of the levels of the various cell wall precursors with and without induction in strains with prototype and non-prototype Tn*1546*-related elements would be required to confirm this hypothesis.

### 7.5 Conclusions

The results presented in this thesis have demonstrated a heterogeneous GRE population within Scottish hospitals. Amongst the clinical nosocomial isolates, the predominant glycopeptide resistance phenotype is VanA, with the majority being *E. faecium*. PFGE analysis of the nosocomial isolates revealed an outbreak strain of VanA *E. faecium* that was responsible for an outbreak within the renal unit of the Royal Infirmary of Edinburgh in 1995. The same outbreak strain was identified in three isolates from two other hospitals in the Edinburgh/Borders region, suggesting interhospital spread. Discounting this outbreak strain, PFGE analysis revealed a diverse set of GRE isolates, with only small clusters of related isolates being identified. PFGE analysis performed on a collection of glycopeptide-sensitive enterococcal

isolates from hospital and general practice sources also revealed a diverse set of isolates. Notably, however, at least one GSE strain appeared to be endemic within the Royal Infirmary of Edinburgh, and many strains were found in both the hospital and community settings. In addition, the two predominant strains of GSE identified were closely related to GRE strains that were part of the outbreak in 1995, and may have arisen from these GRE strains losing their glycopeptide resistance.

A common transferable genetic element was identified in the majority of VanA *E. faecium* isolates on the basis of conjugation experiments, PFGE analysis and hybridization studies. Whilst attempts at plasmid extraction were largely unsuccessful, the combination of PFGE analysis and hybridization studies suggested the element was, indeed, a large plasmid of approximately 160-kb.

Tn*1546*-related elements within the VanA isolates studied showed the same degree and types of variation as described previously. The majority of isolates harboured non-prototype Tn*1546* elements that contained IS*1542* and an IS*1216V*-like insertion within the *orf2-vanR* and *vanX-vanY* intergenic regions respectively. These same Tn*1546*-related elements had substantial variation within the *orf1/orf2* region of the VanA transposon. Whilst the exact nature of this variation was not ascertained, it was at least partly due to the insertion of an IS*1216V*-like element that was accompanied by deletions. Varying geographical distribution of Tn*1546* types was described, as was instability of Tn*1546* variation, both within clonally-related isolates and in isolates during prolonged cold storage. Despite this instability, the value of Tn*1546* typing as an epidemiology tool in localized studies was highlighted.

A hybrid promoter within non-prototype Tn*1546*-related elements was identified, being generated by the insertion of IS*1542* at nucleotide position 3932. The hybrid promoter supplements, rather than replaces, the native *vanR* promoter, and leads to

moderate-level constitutive expression of the *van* resistance genes, as demonstrated by VanX activity in the absence of induction. The presence of this hybrid promoter and constitutive expression of the *van* genes is associated with elevated teicoplanin resistance, although the elevated resistance is, to an extent, media-dependent. The mechanism behind the elevation in teicoplanin resistance is unclear, but may be due to the more rapid and complete elimination of D-Ala-D-Ala-terminating cell wall precursors, and the differing abilities of vancomycin and teicoplanin to act against residual D-Ala-D-Ala-terminating precursors.

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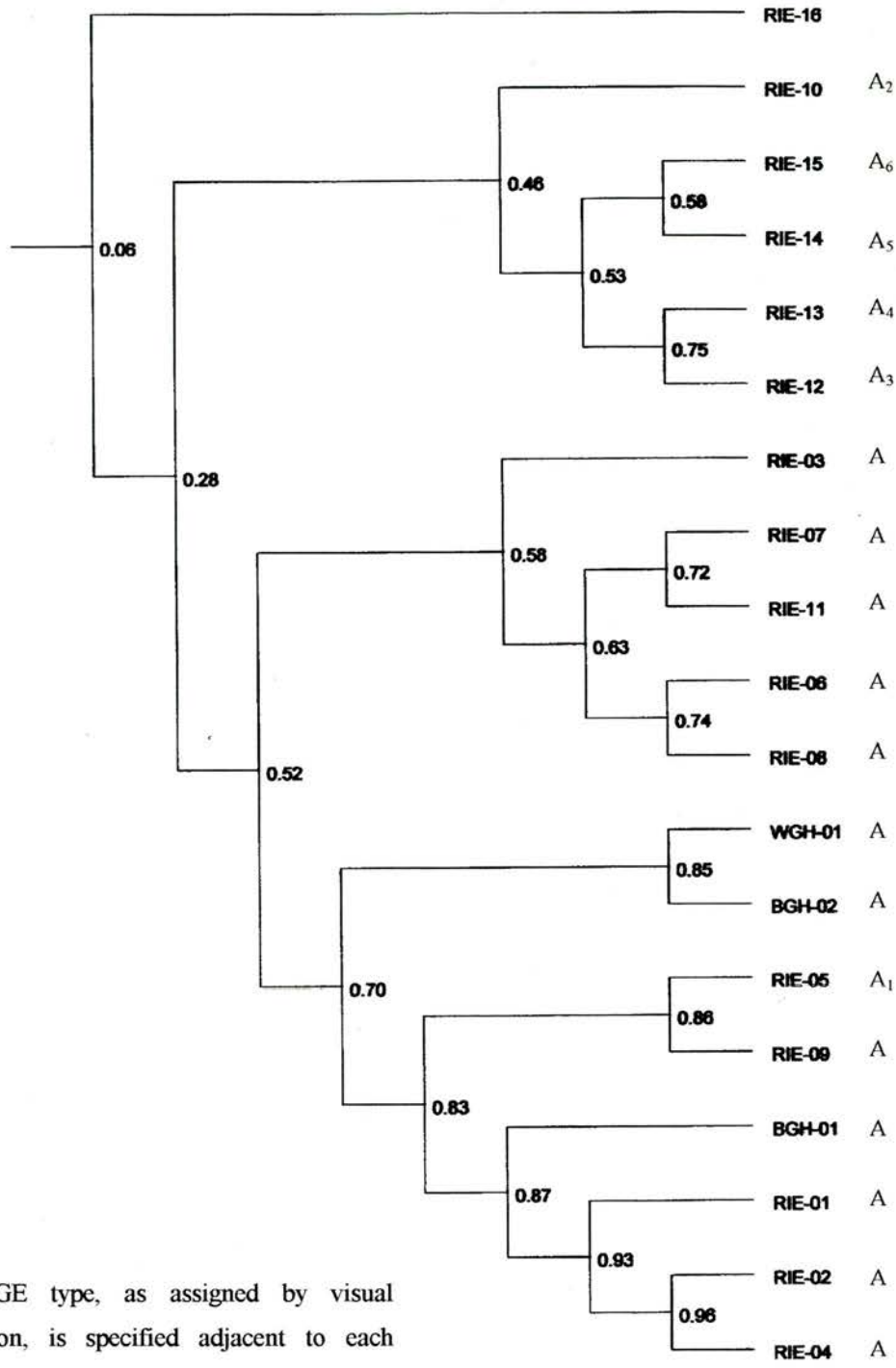
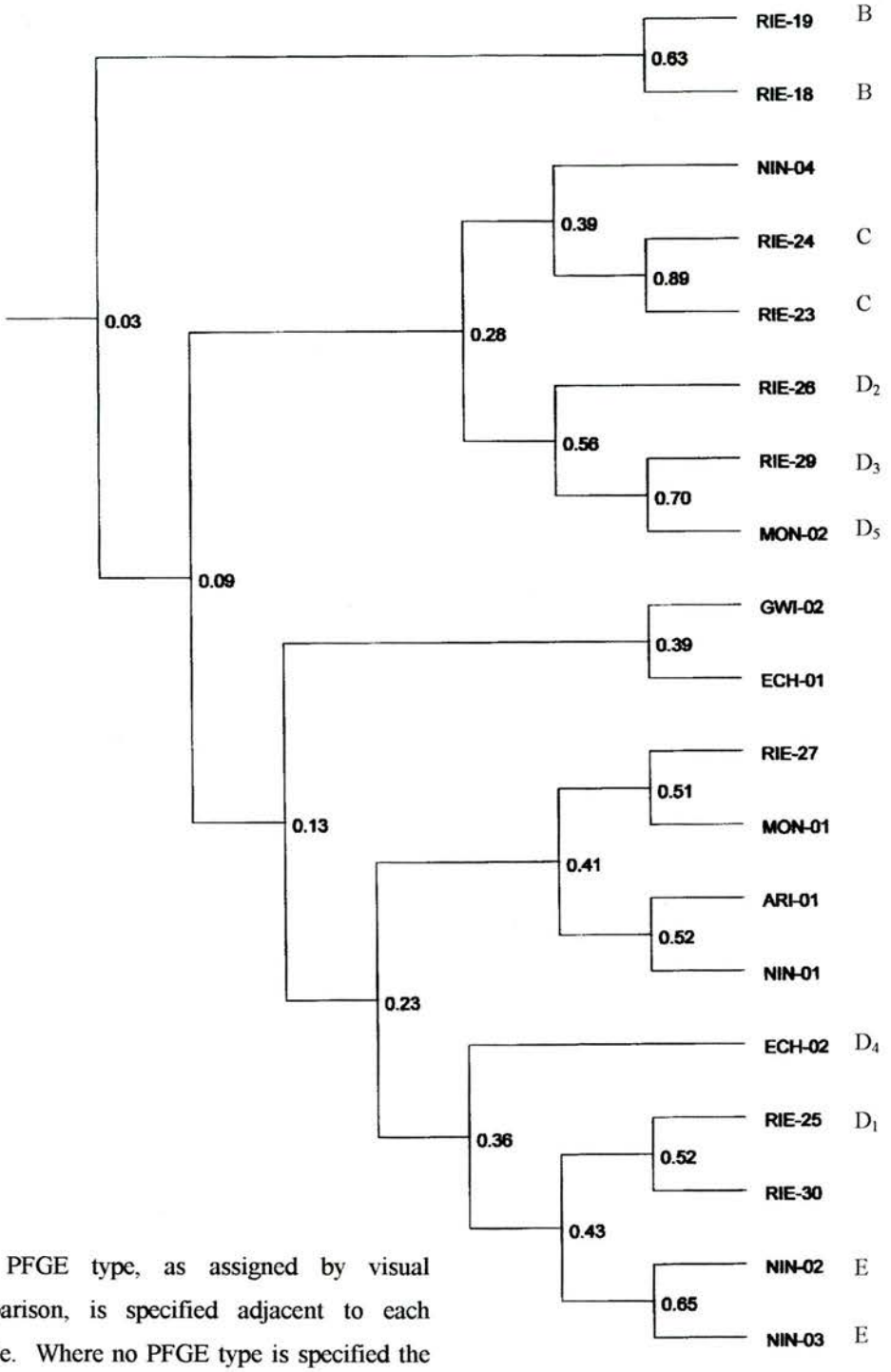
# Appendices

## Appendix A: Phylogenetic tree analysis of PFGE patterns depicted in figure 3.2(a)



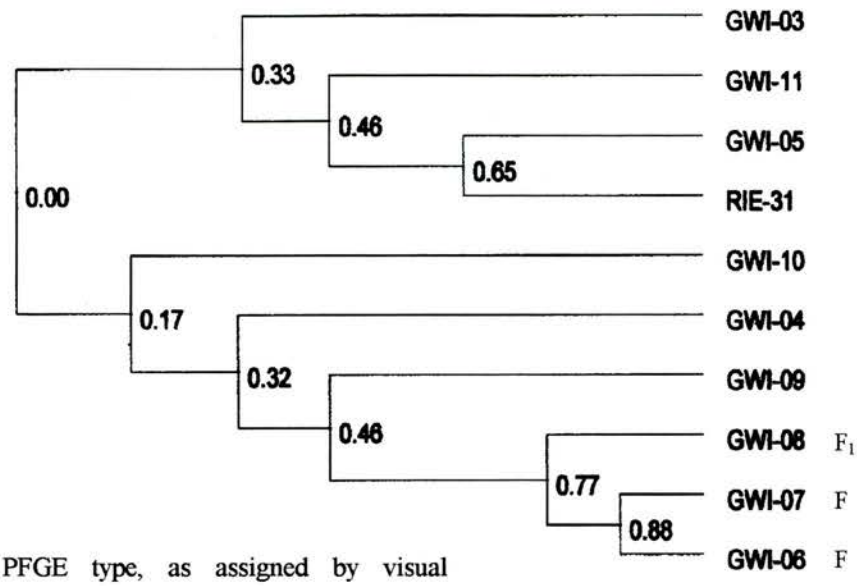

The PFGE type, as assigned by visual comparison, is specified adjacent to each isolate. Where no PFGE type is specified the isolate is unrelated to any other.

## Appendix B: Phylogenetic tree analysis of PFGE patterns depicted in figure 3.2(b)




The PFGE type, as assigned by visual comparison, is specified adjacent to each isolate. Where no PFGE type is specified the isolate is unrelated to any other.

## Appendix C: Phylogenetic tree analysis of PFGE patterns depicted in figure 3.2(c)




The PFGE type, as assigned by visual comparison, is specified adjacent to each isolate. Where no PFGE type is specified the isolate is unrelated to any other.

**Appendix D: Teicoplanin MICs for a selection of VanA GRE harbouring prototype and non-prototype Tn*1546*-related elements**

| | Media used for sensitivity testing | | |
|---|---|---|---|
| **Isolate** | **ISTA** | **MHA** | **BHIA** |
| **Prototype Tn*1546* elements** | | | |
| **ECH-01** | 16 | 64 | 8 |
| **ARI-01** | 32 | 64 | 32 |
| **NIN-01** | 32 | 64 | 32 |
| **NIN-02** | 16 | 16 | 32 |
| **NIN-03** | 16 | 16 | 32 |
| **NIN-04** | 8 | 4 | 16 |
| **GWI-02** | 32 | 64 | 32 |
| **GWI-03** | 4 | 8 | 8 |
| **GWI-04** | 8 | 64 | 16 |
| **GWI-05** | 8 | 8 | 32 |
| **GWI-09** | 16 | 16 | 32 |
| **GWI-10** | 4 | 8 | 16 |
| **GWI-11** | 4 | 8 | 8 |
| **RIE-31** | 4 | 8 | 8 |
| **Non-prototype Tn*1546* elements** | | | |
| **RIE-02** | 64 | 32 | 512 |
| **RIE-03** | 64 | 64 | 256 |
| **RIE-04** | 16 | 64 | 32 |
| **RIE-05** | 128 | 64 | 256 |
| **RIE-06** | 128 | 64 | 512 |
| **RIE-07** | 64 | 32 | 128 |
| **WGH-01** | 128 | 32 | 1024 |
| **RIE-08** | 128 | 64 | 1024 |
| **RIE-09** | 32 | 32 | 256 |
| **BGH-01** | 32 | 32 | 256 |
| **BGH-02** | 32 | 128 | 64 |
| **RIE-11** | 128 | 64 | 512 |
| **RIE-12** | 16 | 32 | 128 |
| **RIE-13** | 16 | 32 | 64 |
| **RIE-15** | 32 | 32 | 256 |
| **RIE-16** | 32 | 32 | 512 |
| **RIE-18** | 64 | 32 | 128 |
| **RIE-19** | 64 | 32 | 32 |
| **RIE-23** | 64 | 32 | 64 |

ISTA – Iso-Sensitest agar; MHA – Mueller-Hinton agar; BHIA – Brain heart infusion agar. Teicoplanin MICs are shown in mg/L.

## Appendix E: Teicoplanin MICs determined with or without pyruvate/lactate supplementation

| ISOLATE | TEICOPLANIN MICs (mg/L) | | | | |
|---|---|---|---|---|---|
| | ISTA | ISTA + 0.1mM sodium pyruvate | ISTA + 0.01mM sodium pyruvate | ISTA + 0.1% lactic acid | ISTA + 0.01% lactic acid |
| **Prototype Tn*1546* elements** | | | | | |
| **ECH-01** | 32 | 64 | 64 | - | 64 |
| **ARI-01** | 32 | 64 | 64 | - | 32 |
| **NIN-01** | 32 | 64 | 64 | - | 32 |
| **NIN-02** | 32 | 64 | 64 | - | 64 |
| **NIN-03** | 32 | 64 | 64 | - | 128 |
| **NIN-04** | 32 | 64 | 64 | - | 64 |
| **GWI-02** | 32 | 64 | 64 | - | 64 |
| **GWI-03** | 16 | 16 | 8 | - | 16 |
| **GWI-04** | 32 | 64 | 32 | - | 32 |
| **GWI-05** | 32 | 64 | 32 | - | 64 |
| **GWI-09** | 32 | 32 | 32 | - | 32 |
| **GWI-10** | 16 | 16 | 32 | - | 32 |
| **GWI-11** | 16 | 16 | 16 | - | 32 |
| **RIE-31** | 8 | 16 | 8 | - | 16 |
| **Non-prototype Tn*1546* elements** | | | | | |
| **RIE-02** | 250 | 250 | 500 | - | 250 |
| **RIE-03** | 250 | 250 | 250 | - | 250 |
| **RIE-04** | 32 | 128 | 128 | - | 128 |
| **RIE-05** | 250 | 250 | 250 | - | 250 |
| **RIE-06** | 250 | 250 | 500 | - | 500 |
| **RIE-07** | 128 | 128 | 250 | - | 500 |
| **WGH-01** | 250 | 250 | 500 | - | 250 |
| **RIE-08** | 250 | 250 | 250 | - | 250 |
| **RIE-09** | 128 | 250 | 250 | - | 250 |
| **BGH-01** | 128 | 128 | 128 | - | 250 |
| **BGH-02** | 64 | 32 | 128 | - | 128 |
| **RIE-11** | 250 | 250 | 500 | - | 500 |
| **RIE-12** | 64 | 64 | 128 | - | 64 |
| **RIE-13** | 128 | 128 | 128 | - | 128 |
| **RIE-15** | 128 | 250 | 250 | - | 128 |
| **RIE-16** | 128 | 250 | 250 | - | 250 |
| **RIE-18** | 128 | 250 | 128 | - | 250 |
| **RIE-19** | 64 | 64 | 64 | - | 64 |
| **RIE-23** | 64 | 64 | 128 | - | 64 |

0.1% lactic acid inhibited bacterial growth.

## Appendix F: API profile, antibiogram, PFGE type and Tn*1546* type of GRE isolates from the outbreak within the renal unit of the Royal Infirmary of Edinburgh

| Isolate | Species | API profile | Antibiotic resistance profile | PFGE | Tn*1546* type |
|---|---|---|---|---|---|
| RIE-01 | FM | 7357511 | Vm, Te, Am, Amp, HLSR | A | B |
| RIE-02 | FM | 7357510 | Vm, Te, HLSR | A | B |
| RIE-03 | FM | 7357511 | Vm, Te, Em, Amp, Tet, HLSR | A | C |
| RIE-04 | FM | 7357511 | Vm, Te, Em, Amp, Tet, HLSR | A | |
| RIE-05 | FM | 7357511 | Vm, Te, Em, Amp, HLSR | $A_1$ | B |
| RIE-06 | FM | 7357511 | Vm, Te, Em, Amp, HLSR | A | B |
| RIE-07 | FM | 7357511 | Vm, Te, Em, Amp, HLSR | A | B |
| RIE-08 | FM | 7357511 | Vm, Te, Em, Amp, Tet, HLSR | A | C |
| RIE-09 | FM | 7357511 | Vm, Te, Em, Amp, Tet, HLSR | A | B |
| RIE-10 | FM | 7357511 | Vm, Te, Em, Amp, Tet, HLSR | $A_2$ | B |
| RIE-11 | FM | 7357511 | Vm, Te, Em, Amp, HLSR | A | D |
| RIE-12 | FM | 7357510 | Vm, Te, Em, Amp | $A_3$ | |
| RIE-13 | FM | 7156511 | Vm, Te, Em, Amp, Tet, HLSR | $A_4$ | |
| RIE-14 | FM | 7557511 | Vm, Te, Em, Amp, Tet, HLSR | $A_5$ | |
| RIE-15 | FM | 7357711 | Vm, Te, Em, Amp, Tet, HLSR | $A_6$ | |
| RIE-16 | FM | 6357510 | Vm, Te, Em, Amp, HLSR | $A_7$ | K |
| RIE-17 | FM | 6357510 | ND | $A_8$ | n/a |
| RIE-18 | FM | 7357711 | Vm, Te, Em, Amp, HLSR | B | B |
| RIE-19 | FM | 7357510 | Vm, Te, Em, HLSR | B | B |
| RIE-21 | FS | 7143711 | ND | $C_1$ | n/a |
| RIE-22 | FS | 7153711 | ND | $C_2$ | n/a |
| RIE-24 | FS | 7173711 | Vm, Te, Em, Amp, HLSR | C | B |
| RIE-25 | FS | 7143311 | Vm, Te, Em, Cm, Tet, HLSR, HLGR | $D_1$ | E |
| RIE-26 | FS | 5143311 | Vm, Te, Em, Cm, Tet, HLSR, HLGR | $D_2$ | |
| RIE-27 | FS | 7143311 | Vm, Te, Em, Amp, Tet, HLSR | | |
| RIE-28 | FS | 5153711 | ND | | n/a |

Antibiotic resistance profile was only established for VanA isolates. ND denotes VanB isolates for which the resistance profile was not determined. Where no Tn*1546* type is stated, it could not be assigned due to failure of L-PCR or insufficient yield to enable restriction analysis. 'n/a' denotes not applicable (VanB isolates).

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**ORIGINAL ARTICLES**

# Epidemiology and control of vancomycin-resistant enterococci (VRE) in a renal unit

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**Summary:** This study reports an outbreak of infection and colonization caused by vancomycin-resistant enterococci (VRE) in the renal service of a large teaching hospital. The polymerase chain reaction and pulsed-field gel electrophoresis were used to study the epidemiology of 26/34 strains of vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium* from the outbreak in comparison with five strains from other hospitals in Edinburgh and the Borders, and three from other wards in the Royal Infirmary. The study revealed a heterogeneous population of vancomycin-resistant *E. faecalis*. Over 60% of *E. faecium* isolates had matching pulsed-field gel electrophoresis patterns and all of these were of VanA phenotype. These results suggest that clonal spread of VanA phenotype *E. faecium* within and possibly between hospitals is the major vancomycin-resistant enterococcal problem in Edinburgh. Screening of patients and isolation of colonized and infected patients appear to have been successful in controlling the spread of VRE.

*Keywords*: Vancomycin-resistant enterococci (VRE); bacterial resistance; renal unit.

## Introduction

Enterococci are frequent causes of urinary tract infections,[1] bacteremia and endocarditis.[2] *Enterococcus faecalis* is most commonly isolated but *Enterococcus faecium* is more inherently resistant to a wide range of antibiotics including penicillin and ampicillin.[3] Vancomycin sometimes in combination with gentamicin is often the treatment of choice for serious enterococcal infections;[4] but the emergence and spread of glycopeptide and aminoglycoside resistance has made therapy considerably more difficult.

Four classes of vancomycin resistance have been identified based on the level of resistance and whether resistance is constitutive or inducible.[5] The VanA phenotype is characterized by high-level, inducible resistance to vancomycin and teicoplanin whilst VanB phenotypes show inducible moderate resistance to vancomycin but remain susceptible to teicoplanin.[5,6] VanC resistance, characterized by constitutive low-level resistance to vancomycin, is a property

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of *Enterococcus gallinarum* and *Enterococcus casseliflavus*[5,7] both of which are rarely encountered clinically. The *vanA* gene is frequently plasmid-borne, carried by *Tn1546* whilst *vanC* is regarded as chromosomal.[5] Conjugal transfer of *vanB* has been demonstrated, being associated with both conjugative transposons and plasmids.[8,9] The recently-described VanD phenotype exhibits constitutive resistance to vancomycin and to low levels of teicoplanin.[10]

This paper describes the epidemiology, laboratory characterization and control methods employed to contain an outbreak of VRE infection affecting the renal service of a large teaching hospital.

## Materials and methods

### *Surveillance methods*

Stool samples and sometimes rectal swabs from patients on the affected renal wards were examined weekly until no new cases were detected for three consecutive weeks. Re-admitted patients were screened on admission and weekly thereafter. Samples from appropriate sites were taken from patients thought to be clinically infected. It was not considered necessary to screen new admissions to the unit from other hospitals. Staff were not screened. Environmental samples were not taken.

### *Microbiological methods*

Enterococci were sought in clinical material from infected renal patients and in stools from patients undergoing screening by culture on Columbia base agar (Unipath) supplemented with 5% horse blood and 10 mg/L added neomycin. Two antibiotic discs containing 5 μg and 30 μg of vancomycin were placed in the inoculum well and the plates were incubated at 37°C overnight in air.

All vancomycin resistant, Lancefield group D streptococci were identified to species level using the API 20 Strep system (BioMerieux, Marcy-l'Ftoile, France). MIC tests were performed on Columbia base agar supplemented with 5% horse blood.

Five VRE isolates from other hospitals; two from the City Hospital, Edinburgh, two from the Borders General Hospital, Melrose and one from the Eastern General Hospital, Edinburgh, were included in the study together with three from other wards in the hospital to ascertain whether there was any relationship between these strains and those isolated from renal patients.

Isolates were stored frozen on beads (Pro-Lab diagnostics, Wirral, UK) at −70°C. These were recovered by plating on Columbia base agar containing 5% horse blood. Broth cultures were made in brain–heart infusion broth. All cultures were incubated at 37°C.

### *Polymerase chain reaction (PCR)*

Oligonucleotides specific for internal fragments of the *vanA* and *vanB* genes[11] were synthesized (Oswel DNA service, Southampton). Cells were harvested from 5 mL of an overnight culture; suspended in 20 μL of a 50 mM Tris–HCl (pH 8·0)/10 mM EDTA solution; and placed in a boiling water-bath for 1 min. Five microlitres of the resulting suspension was used in the PCR mixture. The PCR protocol was a modification of that described by Dutka-Malen *et al.*[11] The PCR was performed in a final volume of 100 μL containing the 5 μL cell suspension; 50 pmol of each oligonucleotide primer; 500 μM dATP, dTTP, dCTP, dGTP; 7 mM $MgCl_2$; 10 μL of 10 × PCR buffer (GibcoBRL, Life Technologies Ltd., Paisley); and 2U Taq DNA polymerase (GibcoBRL, Life Technologies Ltd.). The cycles were 94°C for 2 min for the first cycle; 94°C for 1 min, 54°C for 1 min, 72°C for 1 min for the next 24 cycles; and 72°C for 10 min for the last cycle in a Techne thermal cycler (Cambridge). PCR products were run on 1·5% agarose-Tris–acetic–EDTA gel at 100 V. Gels were stained with ethidium bromide, destained in distilled water, and photographed with UV illumination. *E. faecalis* ATCC 51299 and *E. faecalis* NCTC 12697 were used as *vanB* positive and negative controls, respectively. All VRE isolates were screened with a mixture of *vanA* and *vanB*-specific PCR primers.

The identify of a selection of PCR products

was checked by *Pst*I restriction analysis. Forty microlitres of PCR product was added to 4 μL distilled water and 5 μL of 10 × RE buffer (Promega, Southampton). Ten units of *Pst*I (Promega, Southampton) was added and the samples incubated at 37°C for 1 h. Digests were run on a 4% agarose–Tris–acetic–EDTA gel containing 0·5 μg/mL ethidium bromide.

### *Pulsed-field gel electrophoresis (PFGE)*

The method used for PFGE was based upon that described by Miranda *et al.*[3] Cells from an overnight culture grown in 5 mL brain–heart infusion broth were harvested and suspended in 1·2 mL of PIV solution [1 M NaCl, 10 mM Tris-HCl (pH 7·6)]. Ninety microlitres of this suspension was mixed with 90 μl of 1·6% low-melting preparative grade agarose (Bio-Rad Laboratories Ltd., Herts) and pipetted into small rectangular moulds. The cells in the agarose plugs were lysed overnight by incubation with gentle shaking at 37°C in lysis solution [6 mM Tris–HCl (pH 7·6), 1 M NaCl, 100 mM EDTA (pH 7·5), 0·5% Brij 58, 0·2% deoxycholate, 0·5% sodium lauroyl sarcosine, 20 μg/mL RNase (Sigma Chemical Co., Dorset), and 1 mg/mL lysozyme (Sigma Chemical Co.)]. The plugs were then incubated overnight at 50°C in ESP solution [0·5 M EDTA (pH 9·0–9·5), 1% sodium lauroyl sarcosine, and 50 μg/ml proteinase K (Sigma Chemical Co.)]. The plugs were washed (3 × 30 min) with TE buffer [10 mM Tris–HCl (pH 7·5), 0·1 mM EDTA] and stored at 4°C in TE until restriction.

*Sma*I was used to restrict the DNA; a slice of each plug was placed in 200 μL of 0·1 mg/mL BSA and 25 μL of 10 × RE buffer Y (MBI Fermentas, Immunogen International Ltd., Tyne and Wear). Twenty units of *Sma*I (MBI Fermentas) was added and the plugs were incubated for 6 h at 30°C. The plugs were then washed in TE buffer for 1 h at 37°C before being transferred and sealed into the wells of 1·2% agarose gels in 0·5 × TBE buffer. Electrophoresis was performed on a contour-clamped homogeneous electric fields device (CHEF-DRII; Bio-Rad Laboratories Ltd.). The gel was run for 24 h at 200 V with pulse times increasing from 5 to 40 s. Gels were stained with ethidium bromide for 15 min and destained for 2 × 15 min in distilled water before being photographed with UV illumination.

## Results

### *Description of the outbreak*

The outbreak affected the renal service of a large teaching hospital, accommodated in two ten-bedded areas, with one isolation cubicle and one four-bed area. Rapid intermixing of patients occurred. At frequent intervals, patients were moved for short periods to beds throughout the hospital. The renal high-dependency unit contained six high-dependency beds, including one isolation cubicle and two dialysis stations providing haemodialysis for six to eight patients per day ('bouncing beds'). This unit received patients from other wards and other hospitals. A chronic dialysis unit contained 13 stations each used three times daily so accommodating 75–85 patients per week. Dialysis was also carried out in other high-dependency areas and required constant movement of all grades of staff.

Secure long-term vascular access was impossible in many patients. As a consequence, some patients had multiple infections necessitating removal and replacement of large bore internal jugular and femoral venous catheters. When line-associated sepsis was suspected, patients were treated with parental vancomycin. Vancomycin has a particular appeal because its pharmacokinetics in renal failure means less frequent dosing.

In January 1995, VRE (from urine) were isolated from an elderly maintenance peritoneal dialysis patient who had been an inpatient for over two months. The patient had multiple medical and surgical problems, and had been treated in ITU as well as in a variety of renal wards. The patient was isolated but died eleven weeks later from multiple clinical problems. One week later VRE were isolated from blood culture from another elderly patient who had

recently commenced maintenance haemodialysis. This patient had spent a protracted period in another hospital several weeks previously. The hospital stays of the two patients overlapped and both had received vancomycin for line-associated sepsis or *Clostridium difficile* associated diarrhoea. The second patient died after nine weeks in hospital.

No further cases occurred until 17 March 1995 when VRE were found in blood cultures from a patient undergoing long-term haemodialysis in hospital because of vascular access problems. This patient had been empirically treated with IV vancomycin for line-associated sepsis. The fourth patient was admitted in late March 1995 from Northern Ireland for assessment by the liver unit but died in early April in the renal high-dependency unit. VRE was isolated from blood and urine cultures but was not felt to have contributed to the patient's death.

At this point it was decided to screen all patients (including those attending the chronic dialysis unit). Major concerns were: recent intense clinical activity; the high usage of vancomycin in renal medicine; poor standards of cleaning in wards, sluices and toilet areas, and the risk of spread of VRE to the rest of the hospital.

### Control measures

By 1 May 1995 five patients had been clinically infected and seven were colonized with VRE. Of this group of 12, five were receiving maintenance haemodialysis, two were maintenance peritoneal dialysis patients and five had acute renal failure. A segregated dialysis area was set up for the colonized and infected patients. These patients were not allowed entry to the chronic dialysis unit or to the 'bouncing beds'. The segregated area was also used for outpatients requiring dialysis until they were shown not to be colonized with VRE.

Patients with VRE were nursed in one ward area to which no new VRE-free admissions were permitted. Of necessity, this meant that eventually some patients in this ward were not

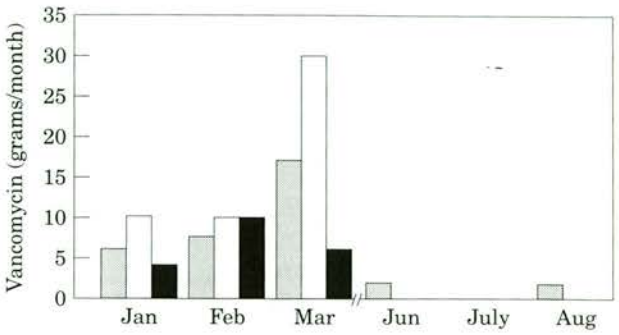


**Figure 1** Vancomycin consumption within the renal unit of Edinburgh Royal Infirmary (1995). (▨) High dependancy unit; (□) Main ward; (■) Chronic dialysis unit.

colonized. Transfers required consultant approval. Discharge to the community was encouraged. Once empty the ward would be cleaned before re-use. A new ward area was opened for medically stable renal patients free from VRE.

Use of glycopeptides (particularly vancomycin) was reviewed (see Figure 1). Metronidazole was instituted as first choice treatment for proven *Clostridium difficile* associated colitis. Antibiotic treatment of proven infection was begun only after consultant approval and reviewed according to clinical response and bacteriological results. A memorandum was issued to all hospital consultants and middle-grade staff indicating the problem in renal medicine and discouraging use of glycopeptides. Following this the hospital-wide usage of vancomycin dropped significantly.

Netilmicin and flucloxacillin were used as the major antistaphylococcal agents. Previous surveillance had indicated that 99% of coagulase-negative staphylococci from renal patients were sensitive to netilmicin. The occasional use of vancomycin for the treatment of CAPD peritonitis was permitted.

Doctors white coats were banned from ward areas. A fresh plastic apron was worn by staff when examining or carrying out procedures on all patients. Gloves were worn for all procedures. Stethoscopes and sphygmomanometers were allocated to individual bed spaces. Hand disinfection between patients

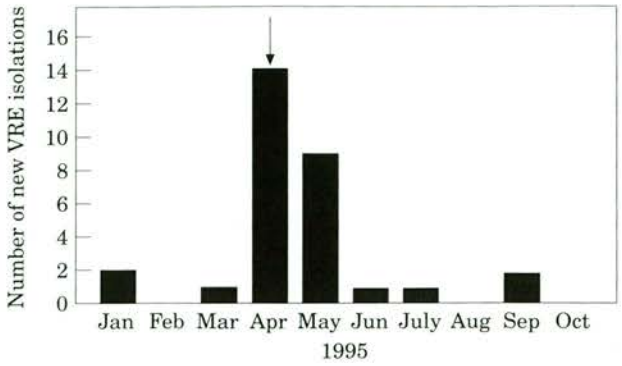


**Figure 2** Graphical representation of the VRE isolates indicating time of isolation (some patients were infected/colonized with more than one strain). Arrow indicates introduction of control measures.

using alcoholic chlorhexidine was rigorously insisted upon. A fresh cleaning contract was negotiated to allow at least thrice daily cleaning of ward toilet and sluice areas. Bedside lockers and major items of equipment were washed down daily with hot water and detergent. The ward washer/disinfectors were checked by engineers. Procedures performed elsewhere in the hospital on renal patients were scheduled at the end of lists to allow routine cleaning thereafter.

Following introduction of these measures three more patients became clinically infected and 10 were found to be colonized. Nine occurred in May, and the remainder sporadically up to the end of September. A graphical representation of the VRE isolates indicating time of isolation and introduction of control measures is shown in Figure 2. None of the patients attending the chronic dialysis unit were colonized.

### *Characteristics of VRE*

In total, 30 isolates were obtained from renal patients infected/colonized with VRE. Unfortunately four were unavailable for further study. The remaining 26 isolates together with three from other ward areas within the hospital and five from other hospitals were evaluated. Some patients had more than one stain identified. Twenty-three of the 34 isolates examined were identified as *E. faecium* (Table I) and eleven as *E. faecalis* (Table II). Fourteen *E. faecium* isolates belonged to one of two API Strep profile groups (7357510 or 7357511). *E. faecalis* isolates demonstrated a more diverse range of API profiles. The minimum inhibitory concentrations (MICs) of vancomycin fell into two groups. (1) 4–32 mg/L; (2) $\geq$512 mg/L. The vancomycin resistance phenotype could be predicted from these values and all were subsequently confirmed by PCR (Figure 3). Twenty-five isolates had MICs of $\geq$512 mg/L and were identified as VanA. Nine isolates had MICs of 4–32 mg/L and were identified as VanB. Eighty-seven percent of *E. faecium* isolates were VanA compared with only 45% of *E. faecalis* isolates. Tables I and II show the resistance type and the API profile of the isolates together with their clinical origin. A selection of PCR fragments had their identity confirmed with restriction enzyme PstI which cuts the vanA PCR fragment once but not the *vanB* fragment.

The criteria described by Tenover *et al.*[12] were used to interpret the PFGE patterns. Isolates were designated indistinguishable if their patterns matched band for band. Patterns which differed by a few bands were considered to indicate closely related isolates which were probably epidemiologically related. Four to six band differences implied that isolates were possibly related; seven or more differences indicated unrelatedness. The 23 *E. faecium* isolates exhibited nine distinct patterns. One was identified as the outbreak pattern (PFGE type A); being shared by 15 different isolates, all of which were VanA (Figure 4). The remaining eight *E. faecium* isolates had distinguishable PFGE patterns although five differed from the outbreak pattern by no more than two or three bands and were accordingly classed as PFGE subtypes of A; $A_1$–$A_5$. Isolates apparently unrelated to the outbreak strain were classed as PFGE types B, $B_1$ and C. Two of the *E. faecalis* isolates had matching PFGE patterns and were classed as PFGE type D with subtypes $D_{1-3}$. Similarly the remaining isolates were designated as types $E_{1-4}$, F and G. The PFGE patterns of the *E. faecalis* isolates are shown in Figure 4 alongside the outbreak pattern (PFGE type A).

**Table I** *Origin and relevant information for vancomycin resistant strains of* Enterococcus faecium.

| Patient ID number | Location | API profile | MIC (mg/L) | Van gene | PFGE type |
|---|---|---|---|---|---|
| 1 | Renal | 6357510 | 1024 | A | $A_4$ |
| 2 | Renal | 7357511 | >1024 | A | A |
| 4 | Renal | 7357510 | >1024 | A | A |
| 5 | Renal | 6357510 | 8 | B | $A_5$ |
| 6 | Renal | 7143311 | >1024 | A | A |
| 9 | Renal | 7357511 | >1024 | A | A |
| 10 | Renal | 7357511 | 1024 | A | A |
| 12 | Renal | 7357711 | 1024 | A | A |
| 13 | Renal | 7357511 | >1024 | A | A |
| 16 | EGH* | 7357510 | >1024 | A | A |
| 17 | Renal | 7357511 | >1024 | A | A |
| 18 | Renal | 7357510 | >1024 | A | A |
| 19 | Renal | 7357510 | >1024 | A | A |
| 20 | Renal | 7357510 | 16 | B | $B_1$ |
| 21 | Renal | 7357711 | >1024 | A | $A_3$ |
| 23 | Renal | 7156511 | >1024 | A | $A_1$ |
| 24 | Renal | 7357511 | >1024 | A | A |
| 25 | Renal | 7357511 | >1024 | A | B |
| 25 | Renal | 7357511 | 512 | A | A |
| 26 | ECH† | 7157511 | 1024 | A | C |
| 27 | Renal | 7557511 | 32 | B | $A_2$ |
| 28 | BGH‡ | 7357511 | >1024 | A | A |
| 29 | BGH‡ | 7357711 | >1024 | A | A |

*Eastern General Hospital
†Edinburgh City Hospital
‡Borders General Hospital
MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis.

**Table II** *Origin and relevant information for vancomycin resistant strains of* Enterococcus faecalis.

| Patient ID number | Location | API profile | MIC (mg/L) | *Van* gene | PFGE type |
|---|---|---|---|---|---|
| 3 | Renal | 7143311 | >1024 | A | $E_1$ |
| 5 | Renal | 5153711 | 4 | B | F |
| 7 | ECH* | 7143311 | >1024 | A | $E_2$ |
| 8 | Renal | 5143311 | 16 | B | $E_3$ |
| 10 | Renal | 7153711 | 4 | B | $D_1$ |
| 11 | Medical | 7173311 | 1024 | A | $D_2$ |
| 12 | Renal | 7173711 | 16 | B | $D_3$ |
| 14 | Transplant | 7173711 | 16 | B | D |
| 15 | G.I./liver | 7173311 | >1024 | A | G |
| 22 | Renal | 7143311 | 1024 | A | $E_4$ |
| 22 | Renal | 7143711 | 16 | B | D |

*Edinburgh City Hospital.
MIC, minimum inhibitory concentration; PFGE, pulsed-field gel electrophoresis.

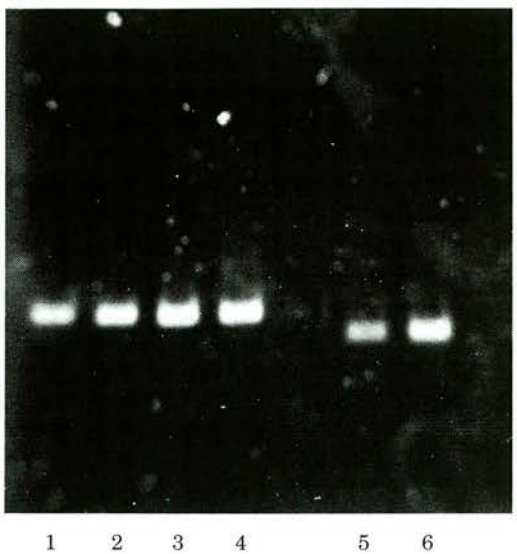


**Figure 3** PCR analysis of vancomycin-resistant enterococci. Lanes 1–4 show PCR product of the *vanA* gene (732 base pairs); lanes 5 and 6 show *vanB* gene PCR products (635 base pairs). On subsequent gels PCR products were run alongside a 100-bp ladder.

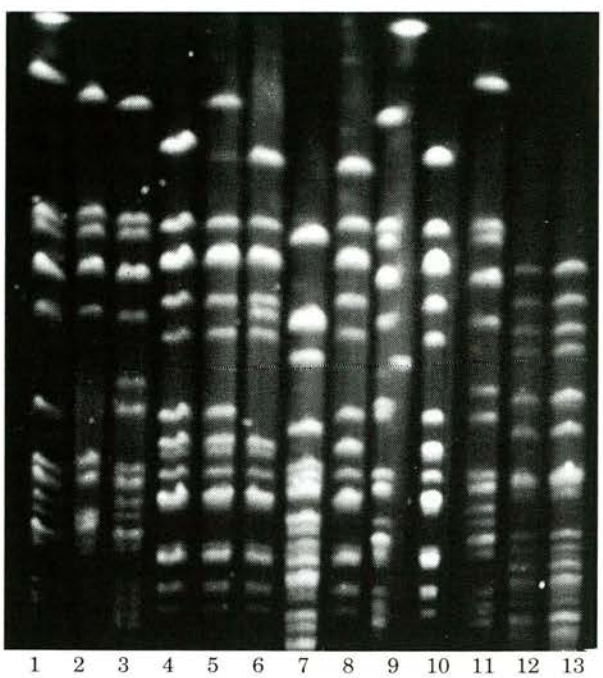


**Figure 4** PFGE patterns of *E. faecalis* and *E. faecium* generated by *Sma*I. Lanes 1–11 show *E. faecalis*; lanes 12 and 13 *E. faecium*. The two E. faecium isolates show the outbreak pattern (type A) as exhibited by 15 VanA *E. faecium* isolates. Lanes 8 and 10 show pattern type D; lanes 4, 5, and 6 show subtypes D1–D3; lanes 1, 2, 3, and 11 were classed as types E1–E4; lanes 7 and 9 as types F and G, respectively.

**Table III** *Total numbers of isolates of VRE obtained from both clinical specimens and screening sampling of renal patients (some patients were infected/colonized with more than one strain).*

| | Infected patients | Colonized patients |
|---|---|---|
| *E. faecium* VanA phenotype | 4 | 12 |
| *E. faecium* VanB phenotype | — | 3 |
| Not studied | 3 | — |
| *E. faecalis* VanA phenotype | 1 | 1 |
| *E. faecalis* VanB phenotype | — | 5 |
| Not studied | 1 | — |

### *Epidemiology in renal patients*

*E. faecium*

*Infected patients:*. Eight patients had clinical infections with VRE. Strains from four were not analysed by PFGE. However, the two initial strains and one strain isolated late in the outbreak were all *E. faecium* and two had MICs of >256 mg/L to vancomycin. It is therefore likely that these were *vanA* strains.

Three other patients were infected with *E. faecium*; all three isolates were *vanA* genotype PFGE type A. One was also infected with a second strain of *E. faecium*, which typed as *vanA* genotype PFGE type B (See Tables I and III).

*Colonized patients:*. Fifteen patients were colonized with *E. faecium*. Twelve strains typed as *vanA* genotype and all were epidemiologically related to PFGE type A. Three patients were colonized with *E. faecium* of *vanB* genotype with PFGE types $A_2$, $A_5$ and $B_1$.

*E. faecalis*

The pattern of colonization or infection with *E. faecalis* was heterogeneous. One patient was infected with *E. faecalis* of *vanA* genotype PFGE type $E_1$. Five patients were colonized with *E. faecalis* of genotype *vanB*. One of the latter was also colonized with *E. faecalis* genotype *vanA* PFGE type $E_4$ and two with *E. faecium* [one with *vanA* genotype PFGE type A; one with *vanB* genotype PFGE type $A_5$ (see Tables I, II and III)].

### *Epidemiology in non-renal patients*

Of the four *E. faecium* isolates obtained from other hospitals three were *vanA* PFGE type A, and one was *vanA* PFGE type C (see Table I). Three of the four vancomycin-resistant *E. faecalis* strains emanated from other wards within the hospital. A patient from the liver transplant unit was colonized with *E. faecalis* of *vanB* genotype. The patient had not been admitted to the renal unit but had been treated by the renal physicians. Another patient from the gastrointestinal liver unit was colonized with *E. faecalis* of *vanA* genotype PFGE type G; but had no known contact with the renal unit or it's staff. A geriatric patient from a medical ward was colonized with *E. faecalis* of *vanA* genotype with PFGE type $D_2$ (see Table II).

## Discussion

The techniques described here have made it possible to evaluate the nature of nosocomial enterococcal outbreaks with ease. The combination of MIC tests and PCR proved to be a rapid and reliable way of establishing the resistance genotype of clinical isolates. When outbreaks occur it is important to have a reliable typing scheme with which to compare isolates, but until relatively recently no such system has been developed for enterococci. The pulsed-field technique described by Miranda *et al.* proved to be reliable and provided very effective discrimination.

API profile numbers alone could not be relied upon to determine the relatedness of isolates. For both *E. faecium* and *E. faecalis*, matching PFGE patterns were exhibited by isolates with different profile numbers, and isolates with the same profile number often gave distinct PFGE patterns.

The cultural technique used in this study was not optimal. Isolation of *E. faecium* from mixed bacterial populations especially during epidemiological surveillance using heavily contaminated faecal specimens is problematic. Ford *et al.*[13] demonstrated that cephalexin–aztreonam–arabinose agar is a more suitable medium, and it has now been adopted for routine use.

No isolates with identical PFGE patterns had different resistance genotypes. All *E. faecium* isolates of the outbreak strain (PFGE type A) were *vanA* genotype. Similarly, the *E. faecalis* isolates from the renal unit (PFGE type D) were all *vanB* genotype. Only within PFGE subtypes were seemingly related strains carrying different genes. This may cast doubt on the relatedness of these supposedly related strains but it may be at least partially explained by the transfer or loss of *vanA* or *vanB*.

The two *E. faecalis* isolates with the type D PFGE pattern, although displaying slightly different API profiles, were otherwise identical and are therefore the same strain; despite being isolated from different areas. No other *E. faecalis* isolates had identical PFGE patterns although many were either closely or possibly related suggesting an epidemiologic relationship (Table II). In general, however, the *E. faecalis* isolates were more heterogeneous than those of *E. faecium*.

The large group of *vanA* genotype *E. faecium* isolates exhibiting the type A PFGE pattern is of particular interest. Within this group are the two isolates from the Borders General Hospital (BGH), Melrose, and the one isolate from the Eastern General Hospital, Edinburgh [the isolate from Edinburgh's City Hospital was unique in this study (PFGE type C)]. The remaining 12 isolates are from the Royal Infirmary. The 15 isolates had very similar, but not identical API profiles. Twelve of the 15 type A strains had one of two API profiles – 7357510 or 7357511. The remaining three isolates had similar profiles; differing only by either positive sorbitol fermentation (7357711) or negative lactose fermentation (7357110). The five *E. faecium* isolates classed as PFGE types $A_1$–$A_5$ were closely related to the outbreak strain and are probably epidemiologically related.

Clonal spread of identical and closely related strains within hospitals is highly possible. More interesting is the possibility of spread between hospitals – particularly in the case of the apparent identity of the Borders General Hospital isolates with those in Edinburgh. Such spread of a single strain is certainly possible as demonstrated by Murray *et al.*[14] when studying isolates from hospitals in five different states.

With *vanA* frequently being plasmid-borne it

may have been expected that a greater diversity of enterococcal strains would be found to be *vanA* positive. However the results suggest that, certainly in Edinburgh, high-level *vanA* genotype resistance is the result of clonal spread of a single strain and not widespread distribution of *vanA* carrying plasmids. Are the *vanA* genes within the isolates from this study largely chromosomally located? Work is now being undertaken to determine the transferability of the *vanA* and *vanB* resistance genes in our isolates. Determining the location of these genes will enhance the understanding of VRE epidemiology.

We believe that the control measures were helpful in containing the outbreak of VRE infection. Specific recommendations for prevention and control of infection with VRE are included in a report of the Hospital Infections Control Practices Advisory Committee of the Centres for Disease Control and Prevention.[15] Screening of staff was not undertaken because no useful action could be taken in the event of finding VRE colonisation. The deaths of four patients were not solely due to VRE infection although this was probably a contributory element in some cases. The VRE strains from BGH were isolated in 1996 from female patients in separate long stay accommodations in Kelso and Cornhill-on-Tweed. There was no known contact with the teaching hospital renal unit. However, without further epidemiological corroboration and examining more isolates from these hospitals, particularly after a screening programme, it is presumptive to say that clonal spread has been shown between these hospitals.

The importance of a multi-faceted approach is emphasized. The last PFGE type A VRE were isolated at the end of September. None have been isolated from clinical material since then, either in the renal wards or elsewhere in the hospital.

## Acknowledgements

We are grateful to Miss Kerry Glendinning, Dr T. Gillespie and Dr M. Brown for the supply of VRE from outside hospitals.

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**Vancomycin-Resistant Enterococci: Epidemiology and control of an epidemic in a Renal Unit.** *W.D. Plant[1], R.J. Winney[1], A. Brown[2], R.H. Paton[2], S.G.B. Amyes[2], G.M. Stevenson[2], and R.S. Miles[2], [1]Dept of Renal Medicine, Royal Infirmary of Edinburgh NHS Trust & [2]Department of Clinical Bacteriology, Edinburgh University, Edinburgh, Scotland.*
Glycopeptide-resistant enterococcus species, especially E. faecalis and E. faecium, are increasingly important causes of nosocomial infection. We report an epidemic affecting 25 patients under the care of a university teaching hospital renal service. Vancomycin-resistant strains (VRE) of E. faecium were cultured in clinical samples from 2 maintenance dialysis patients. Both had multiple medical problems, had spent a protracted period in hospital, and been treated with vancomycin. Two months later, VRE were cultured in clinical samples from 2 other in-patients. Subsequently all in-patients in areas where these patients had been cared for were screened (rectal swab and faecal culture) for VRE. 17/77 (22%) screened patients were colonised and treated as potentially infectious. VRE was isolated in clinical samples from a further 4 patients. 9 patients died whilst colonised/infected, 12 became free from colonisation after a period of time and 4 were discharged into the community. No patient died directly as a result of VRE infection, but it may have contributed as an additional clinical problem in those already seriously ill. Isolates were identified to species level using the API 20 Strep system. MIC tests and PCR was used to evaluate the resistance genotype of each organism. Relatedness was evaluated using restriction endonuclease digestion (RED) and pulsed-flow gel electrophoresis. E. faecium was isolated from 21 patients and E. faecalis from 6 patients (some were colonised with both). The outbreak strain was identified as an E. faecium RED type A with VanA genotype. In the majority of cases this or a related strain was isolated suggesting that the epidemic resulted from clonal spread of this strain. All colonised patients were isolated within a single ward area. Segregated dialysis facilities were provided. Patient transfer between clinical areas was restricted. The frequency of cleaning of surfaces and toilet areas was increased. The use of parenteral and oral vancomycin was severely curtailed. Rigorous attention was paid to hand-washing and wearing plastic aprons. With this approach the epidemic was contained and no new cases have developed in the following 18 months.

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# Isolation and Characterization of Glycopeptide-Resistant Enterococci from Hospitalized Patients over a 30-Month Period

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**In February 1996, a Hospital Infection Control Practices Advisory Committee-style screening program was commenced to isolate and subsequently characterize glycopeptide-resistant enterococci (GRE) from patients at a hospital trust in Glasgow, Scotland. Over the next 30 months, GRE were isolated from 154 patients. GRE were isolated from patients in traditionally high-risk areas such as the renal unit and intensive care unit and also in areas considered to be lower risk, including medical wards and associated long-stay geriatric hospitals. The majority (90%) of isolates were *Enterococcus faecium vanB*. The remaining isolates consisted of seven *E. faecalis* (*vanA*), three *E. gallinarum* (*vanC*), and a further six *E. faecium* (five *vanA*, one both *vanA* and *vanB*) isolates. Analysis of *Sma*I-digested DNA by pulsed-field gel electrophoresis revealed that 34 of 40 (85%) VanB *E. faecium* isolates were identical or closely related, while 11 of 13 (85%) VanA GRE were distinct. High-level aminoglycoside resistance was seen in less than 8% of isolates. VanB *E. faecium* isolates were almost uniformly resistant to ampicillin and tetracycline. In this study, GRE have been isolated over a prolonged period from a broad range of patients. Glycopeptide resistance within the study hospital trust appeared to be mainly due to the clonal dissemination of a single strain of *E. faecium* VanB.**

Following their initial discovery in the United Kingdom and France in 1986 (15, 26), glycopeptide-resistant enterococci (GRE) have now spread worldwide, with the percentage of enterococcal infections in the United States resistant to vancomycin rising from 0.3% in 1989 to 7.9% in 1993 (4). A number of outbreaks of colonization and infection with GRE have been described. VanA-type *Enterococcus faecium* strains typically predominate in clinical areas such as transplantation and oncology units and the intensive care unit (ICU) (3, 7, 9, 19, 21).

The Hospital Infection Control Practices Advisory Committee (HICPAC) of the Centers for Disease Control and Prevention has published recommendations for prevention of the spread of glycopeptide resistance (12) which, although published in the United States, are believed to be relevant in the United Kingdom also (10). The recommendations include screening of enterococcal isolates for vancomycin resistance and establishment of a fecal screening program to detect patients with intestinal colonization with GRE. It is also recommended that control efforts be intensified once GRE are known to be present within a hospital, particularly in high-risk areas such as transplantation and oncology units and the ICU.

An isolate of GRE was obtained from the routine culture of a femoral line tip from a renal unit patient in the ward of a Scottish hospital trust in October 1995. In response to this, all enterococcal isolates from clinical specimens were examined for glycopeptide resistance. A fecal screening program was also established and involved thrice-weekly screening of all ICU patients and routine screening of all fecal specimens or rectal swabs submitted to the laboratory.

This paper presents the findings of this program over a 30-month period.

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## MATERIALS AND METHODS

**Hospital setting.** The study hospital trust is a tertiary referral center that comprises two major hospitals with a combined total of 1,100 beds, together with three smaller, long-stay geriatric hospitals. One of the major hospitals has a 33-bed renal unit located in two adjacent wards on a single floor that comprises a mixture of single- and four-bed rooms. An eight-bed ICU is located two floors below.

**Fecal screening program.** Routine screening of all fecal specimens submitted to the laboratory was instituted in February 1996. Additionally, rectal swabs were taken thrice weekly from all patients on the ICU. Fecal suspensions or rectal swabs were inoculated directly onto esculin azide agar base (Oxoid, Basingstoke, United Kingdom) supplemented with 6 μg of vancomycin (Sigma, Poole, United Kingdom) per ml and with 10 μg of colistin sulfate per ml and 15 μg of nalidixic acid per ml (both from Oxoid). The plates were incubated for 18 h in air at 37°C. Isolates that were esculin positive, catalase negative, gram-positive cocci, and vancomycin resistant by the E-test were tentatively identified as GRE.

**Identification.** Clinical enterococci and those from the fecal screens were identified to the species level in the laboratory with the API 20 STREP system (Biomerieux, Marcy l'Etoile, France). The identities of all isolates were confirmed by PCR and biochemical tests (see Table 1).

PCR amplification of intergenic rRNA spacer regions (ITS-PCR) was based on the methods of Jensen et al. (13) and Tyrrell et al. (25) with primers L1 (5′-CAAGGCATCCACCGT) and G1 (5′-GAAGTCGTAACAAGG). Amplification was performed with a Hybaid Omn-E thermal cycler with 20 pmol of each primer, 100 μM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.1% Triton X-100. Template DNA was prepared from whole cells by mixing 200 μl of a culture grown overnight in brain heart infusion broth (Oxoid) with 800 μl of water and boiling for 10 min. Following centrifugation, 5 μl of supernatant was added to each PCR mixture, and the final volume was adjusted to 50 μl. The PCR mixture was heated to 95°C for 5 min, and 1 U of BioTaq DNA polymerase (Bioline, London, United Kingdom) was added. Amplification conditions were 94°C for 1 min, 55°C for 7 min, and 72°C for 2 min for a total of 25 cycles, followed by an additional 7 min at 72°C.

Arabinose fermentation was detected in a 1% (wt/vol) sugar solution in Andrade's peptone water. The identities of *E. gallinarum* and *E. casseliflavus* strains were confirmed by PCR amplification of the *vanC* ligase gene specific for each organism (6).

**Antimicrobial susceptibility.** All isolates were tested for susceptibility to vancomycin, ampicillin, gentamicin, and streptomycin (all from Sigma), teicoplanin (Marion Merrell, Uxbridge, United Kingdom), and ciprofloxacin (Bayer, Newbury, United Kingdom) by an agar incorporation method in accordance with the British Society for Antimicrobial Chemotherapy guidelines (28).

Glycopeptide-resistant isolates were characterized phenotypically on the basis of susceptibility levels to vancomycin and teicoplanin. Isolates were defined as having a VanA phenotype if the vancomycin MIC was ≥64 μg/ml and the tei-

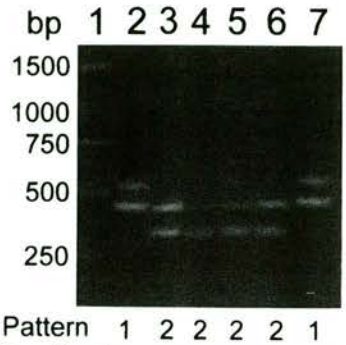


FIG. 1. ITS-PCR profiles of enterococcal species. Lanes: 1, Generuler 1-kb DNA ladder (MBI Fermentas); 2, *E. faecium* ATCC 19434; 3, *E. faecalis* ATCC 19433; 4, *E. casseliflavus* ATCC 25788; 5, *E. gallinarum* ATCC 49573; 6, strain G-089 (*E. faecalis*); 7, strain G-090 (*E. faecium*). The image was generated with Adobe Photoshop, version 4.0.

coplanin MIC was ≥2 μg/ml. For isolates with a VanB phenotype the vancomycin MIC was ≥8 μg/ml and the teicoplanin MIC was <2 μg/ml.

The MIC breakpoints for susceptibility to an antibiotic were as follows: ampicillin, ≤8 μg/ml; tetracycline, ≤1 μg/ml; and ciprofloxacin, ≤4 μg/ml. The numbers of isolates with high-level gentamicin resistance (MICs, >500 μg/ml) and high-level streptomycin resistance (MICs, >2,000 μg/ml) were determined. Cell suspensions were tested for β-lactamase production in a solution of 20 μg of nitrocefin per ml.

**PCR amplification of glycopeptide resistance elements.** Glycopeptide resistance determinants *vanA*, *vanB*, *vanC1*, and *vanC2-vanC3* were detected by multiplex PCR with the primers described by Dutka-Malen et al. (6). Amplification reactions were performed in 50-μl volumes containing 20 μl of template (prepared from boiled whole cells as for ITS-PCR), 20 pmol of each primer, 200 μM deoxynucleoside triphosphates, 3 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8), 0.01% Tween-20, and 1 U of BioTaq DNA polymerase. The reaction mixtures were denatured at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min.

**PFGE typing.** Genomic DNA was prepared in agarose plugs as described by Murray et al. (20). DNA was digested with 20 U of *Sma*I (Promega, Madison, Wis.) for 6 h at 25°C and was electrophoresed with the contour-clamped homogenous electric fields device (CHEF-DRII; Bio-Rad, Hercules, Calif.). The switch interval was ramped from 5 to 35 s over a 24-h period at 6 V/cm². A pulsed-field gel electrophoresis (PFGE) type was assigned to each strain in accordance with the criteria outlined by Tenover et al. (23).

**Control strains.** *E. faecium* NCTC 12202 (*vanA*), *E. faecium* ATCC 19434 (glycopeptide susceptible), *E. faecalis* ATCC 51299 (*vanB*), *E. faecalis* ATCC 19433 (glycopeptide susceptible), *E. gallinarum* ATCC 49573 (*vanC1*), and *E. casseliflavus* ATCC 25788 (*vanC2*) were used as control strains.

## RESULTS

**Isolates.** Between February 1996 and July 1998, GRE were obtained from 154 patients. Only the initial isolate from each patient was used for further study. The majority of isolates (134 of 154) were obtained from the two major hospitals.

Thirty-six isolates were from clinical specimens, including 21 from urine, 10 from wounds, 3 from continuous ambulatory peritoneal dialysis effluents, and 2 from blood cultures. Twenty nine (80.5%) of the 36 clinical GRE were from patients in the primary hospital unit, of which 17 were renal unit patients. A single clinical GRE isolate was recovered from an ICU patient during the study period. Two isolates were from patients in the long-stay geriatric hospitals. These patients represented 0.87% of the 4,130 patients from whom clinically significant enterococci were isolated by the laboratory during the study period.

The remaining 118 GRE were from 8,549 patients screened for fecal carriage (carriage rate, 1.4%). Of these, 23 were recovered from 1,117 ICU patients (carriage rate, 2.1%), 36 were recovered from 316 renal unit patients (11.5%), and 6 were recovered from 153 oncology and hematology unit patients (1.2%). A further 45 were isolated from 6,843 patients (0.7%) in the two major hospitals, including patients from surgical and medical wards, patients from the obstetrics unit, and outpatients. The final eight fecal isolates were recovered from 124 patients (6.5%) within the long-stay geriatric hospitals. There were no apparent associations between colonization with GRE and the clinical indication for the submission of fecal specimens.

TABLE 1. Tests used for differentiating between *E. faecalis*, *E. faecium*, *E. gallinarum*, and *E. casseliflavus* species groups

| Test | Test result | | | |
|---|---|---|---|---|
| | *E. faecalis* | *E. faecium* | *E. gallinarum* | *E. casseliflavus* |
| ITS-PCR | | | | |
| Pattern 1 | − | + | − | − |
| Pattern 2 | + | − | + | + |
| Arabinose fermentation | − | + | + | + |
| Van PCR | | | | |
| *vanC1* | − | − | + | − |
| *vanC2* | − | − | − | + |

**Identification.** The use of ITS-PCR with primers L1 and G1 to produce unique patterns of bands for different enterococcal species has been described previously (25). In our hands, these primers amplified only two distinct patterns of bands from isolates collected for this study and control strains (Fig. 1). Isolates of *E. faecium* gave two bands of approximately 430 and 520 bp (pattern 1). A single distinct pattern (pattern 2), with bands of approximately 295 and 420 bp, was produced by *E. faecalis*, *E. gallinarum*, and *E. casseliflavus* isolates. Thus, species identity was determined by a combination of ITS-PCR, arabinose fermentation, and PCR amplification of *van* genes, as outlined in Table 1. By using these criteria, 144 (93.5%) isolates were identified as *E. faecium*, 7 (4.5%) were identified as *E. faecalis*, and 3 (2%) were identified as *E. gallinarum*. No *E. casseliflavus* isolates were obtained.

The identities of the isolates determined by these tests did not always correlate with the identifies determined with the API 20 STREP system (Table 2). The API 20 STREP kit failed to identify any of the three *E. gallinarum* strains. Furthermore, 29 strains of *E. faecium* were incorrectly identified as *E. casseliflavus* by the API 20 STREP system.

**Characterization of glycopeptide resistance elements.** The Van phenotype of each isolate was determined on the basis of its levels of susceptibility to vancomycin and teicoplanin (Table 3). All seven *E. faecalis* isolates had a VanA phenotype. The majority, 138 of 142 (97%), of *E. faecium* strains were VanB, while the remaining 6 isolates had susceptibility levels that

TABLE 2. Comparison of species identification methods for enterococcal isolates

| Species[a] | No. of strains identified with API 20 STREP system[b] | | | | |
|---|---|---|---|---|---|
| | *E. faecalis* | *E. faecium* | *E. gallinarum* | *E. casseliflavus* | Total |
| *E. faecalis* | 6 | 1 | 0 | 0 | 7 |
| *E. faecium* | 1 | 114 | 0 | 29 | 144 |
| *E. gallinarum* | 0 | 1 | 0 | 2 | 3 |
| *E. casseliflavus* | 0 | 0 | 0 | 0 | 0 |
| Total | 7 | 116 | 0 | 31 | 154 |

[a] Species determined by using the criteria described in Table 1.
[b] API 20 STREP profiles analyzed by using APILAB, version 5.1, software.

TABLE 3. In vitro susceptibilities of *E. faecalis* and *E. faecium* isolates to various antimicrobial agents

| Organism (Van phenotype) and antimicrobial agent | MIC (μg/ml)[a] | | | No. (%) resistant |
|---|---|---|---|---|
| | 50% | 90% | Range | |
| *E. faecalis* (VanA) ($n$ = 7) | | | | |
| Vancomycin | 128 | 512 | 64–512 | 7 (100) |
| Teicoplanin | 8 | 64 | 4–64 | 7 (100) |
| Ampicillin | 2 | 4 | 1–4 | 0 (0) |
| Tetracycline | 128 | >128 | 64–>128 | 7 (100) |
| Streptomycin | 512 | >2,048 | 512–>2,048 | 2 (28.6) |
| Gentamicin | 64 | >1,024 | 32–>1,024 | 1 (14.3) |
| Ciprofloxacin | 1 | 1 | 0.5–1 | 0 (0) |
| *E. faecium* (VanA) ($n$ = 6[b]) | | | | |
| Vancomycin | 256 | >256 | 128–>256 | 6 (100) |
| Teicoplanin | 32 | >128 | 8–>128 | 6 (100) |
| Ampicillin | 32 | >256 | 0.5–>256 | 5 (83.3) |
| Tetracycline | 0.25 | 128 | <0.25–128 | 1 (16.7) |
| Streptomycin | 256 | >2,048 | 128–>2,048 | 1 (16.7) |
| Gentamicin | 32 | >1,024 | 16–>1,024 | 1 (16.7) |
| Ciprofloxacin | 2 | >128 | 0.5–>128 | 2 (33.3) |
| *E. faecium* (VanB) ($n$ = 138) | | | | |
| Vancomycin | 16 | 16 | 8–128 | 138 (100) |
| Teicoplanin | 0.5 | 0.5 | <0.25–1 | 0 (0) |
| Ampicillin | 128 | 128 | 1–256 | 137 (99.3) |
| Tetracycline | 32 | 64 | 0.25–64 | 134 (97.1) |
| Streptomycin | 256 | 256 | 64–>2,048 | 5 (3.6) |
| Gentamicin | 64 | 64 | 32–>1,024 | 1 (0.7) |
| Ciprofloxacin | 2 | 4 | 0.5–32 | 4 (2.9) |

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.
[b] Includes an *E. faecium* strain with both *vanA* and *vanB* ligase genes.

suggested a VanA phenotype. The PCR-determined genotypes were consistent with the phenotypes for all but one isolate. One strain of *E. faecium* with a VanA phenotype produced both *vanA* and *vanB* PCR products. A *vanC1* gene was amplified from the three *E. gallinarum* isolates.

Interestingly, the VanB *E. faecium* strains showed low-level resistance to vancomycin, and the vancomycin MIC was greater than 32 μg/ml for only one isolate.

**Antimicrobial susceptibility.** Strains were tested for susceptibility to ampicillin, tetracycline, streptomycin, gentamicin, and ciprofloxacin (Table 3). *E. faecium* isolates were almost uniformly resistant to ampicillin, while *E. faecalis* isolates remained susceptible. Twenty isolates for which ampicillin MICs covered a wide range were tested for β-lactamase activity. None produced a detectable β-lactamase enzyme. Of the VanB *E. faecium* isolates, 97% were resistant to both ampicillin and tetracycline. Overall, 4% of isolates were resistant to ciprofloxacin. High-level aminoglycoside resistance was seen in less than 8% of isolates, and no isolate was resistant to both streptomycin and gentamicin. The three *E. gallinarum* strains were susceptible to all antibiotics tested.

**Typing.** The 13 VanA GRE were typed by PFGE. All *E. faecalis* isolates had distinct PFGE patterns. Of the six *E. faecium* isolates, three were distinct types and three were identical. The three identical isolates were recovered from fecal screens for patients located in a single four-bed room within the renal unit over a 2-week period in June and July 1997, suggesting that person-to-person spread had occurred.

Forty VanB *E. faecium* isolates were selected for typing, including both fecal and nonfecal isolates from the 30-month period. Thirty-five isolates were from the primary hospital unit (from which 70% of GRE were isolated) and comprised 9 nonfecal isolates from the renal unit and ICU and 26 fecal isolates (23 from the renal unit and ICU, 3 from other wards). Four GRE were isolated from patients in the second hospital unit, and one was from a patient in a geriatric hospital. Twenty-eight of 40 (70%) VanB *E. faecium* isolates had identical PFGE patterns, including both fecal and nonfecal isolates from the two major hospitals. A further six isolates were closely related (two or three band differences), including the isolate from the patient in the geriatric hospital. Five isolates were possibly related (four to six band differences), and one (a fecal isolate from the renal unit) was unrelated.

## DISCUSSION

GRE have become an increasing problem in hospitals from the standpoint of nosocomial infection and infection control. HICPAC has published guidelines on the control of GRE within hospitals, and these recommend the routine screening of enterococcal isolates for resistance to vancomycin and intensified fecal screening to detect patients with gastrointestinal colonization. In this study the findings from such a screening program are presented. These differ from many other studies of colonization with GRE in that they are not restricted solely to patients in high-risk areas over short time periods.

The vancomycin-containing indicator medium used in this study proved useful for the isolation of GRE from fecal specimens. Identification to the species level proved more problematic. Other investigators have reported discrepancies between the identities obtained with the API 20 STREP system and by genotypic methods (16, 27). In this study, the API 20 STREP kit was particularly unreliable at distinguishing *E. faecium* and *E. casseliflavus* isolates. The epidemiological and infection control implications of enterococci with intrinsic (*vanC*) and acquired (*vanA*, *vanB*) resistance to vancomycin are very different, with intrinsically resistant species failing to demonstrate person-to-person spread (24). Failure to correctly identify patients colonized with intrinsically resistant strains entails subjecting them to unnecessary infection control measures. Reanalysis of the API 20 STREP profiles on uprated software correctly identified all strains of *E. faecium* but was still unable to identify two of the three strains of *E. gallinarum*. It would appear that accurate differentiation of intrinsically glycopeptide-resistant species from those with acquired resistance during screening requires a molecular biological methodology such as PCR.

One such method uses amplification of species-specific *ddl* ligase genes to identify *E. faecalis* and *E. faecium* (6). This method appears to be particularly useful as it can be performed in combination with amplification of glycopeptide resistance elements. In this study, we assessed the ITS-PCR method, which has been reported to give distinct bands for a number of enterococcal species, in addition to *E. faecalis* and *E. faecium* (25), for species identification. In our hands, this method could be used to identify *E. faecium* isolates, but further tests were required to distinguish *E. faecalis* from the intrinsically resistant species.

Although only 12% of isolates of GRE in the United Kingdom have the *vanB* genotype (18), the predominance of the *vanB* genotype within a hospital setting has been reported previously (2, 17, 22). However, the low level of vancomycin resistance present within the *vanB* strains (MIC at which 90% of isolates are inhibited, 16 μg/ml) in this study is unusual and may be partially explained by the lower concentration of vancomycin in the selective medium than that used in earlier

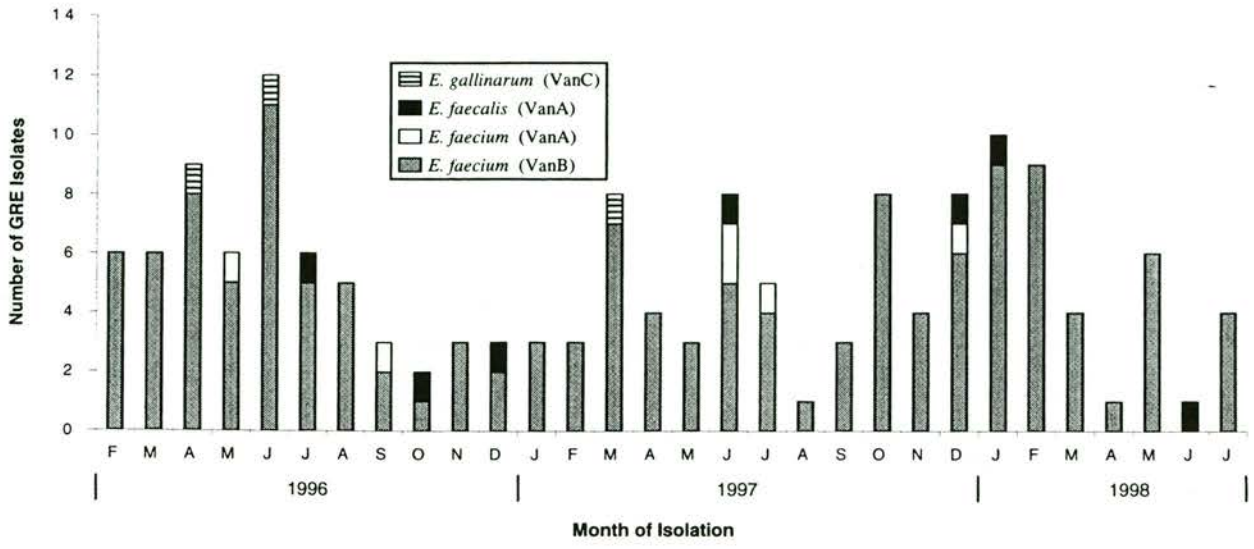


FIG. 2. Isolation of GRE from a Scottish hospital trust over a 30-month period.

studies. The incidence of high-level gentamicin resistance was also low, being found only in a single strain. The rate of high-level gentamicin resistance found in this study (0.7%) is much lower than rates of 6.7 to 13.4% found among all species of enterococci, both vancomycin resistant and sensitive, in the United Kingdom (11). The retention of sensitivity to teicoplanin is fortuitous in that it provides a synergistic therapeutic option in combination with an aminoglycoside should the need arise (14). All *E. faecalis* isolates retained sensitivity to ampicillin, giving an alternative therapeutic option.

The resistance genotype determined by PCR was consistent with the phenotype for all but one isolate, despite reports of mismatching in previous studies (1, 5, 8, 16). There does, however, exist an area of overlap between MICs at the bottom of the range for VanB strains (an area into which most of the VanB study isolates fell) and those at the top of the range for VanC strains, making distinction by phenotypic methods between intrinsic and acquired resistance impossible for certain strains. The failure of phenotypic methods of identification and antimicrobial sensitivity testing to distinguish between acquired and intrinsic resistance consequently necessitates the use of a molecular biological method for accurate differentiation of all strains.

The carriage of GRE described in this study gives a much fuller picture than that often obtained, given the prolonged study period and broad area screened. GRE colonization occurred throughout the study period among patients admitted to the renal unit and ICU, areas traditionally regarded as high risk (12). However, this may reflect the regular nature of the screening protocol in these areas. Among patients in the hematology and oncology units colonization was infrequent in this study, despite frequent use of glycopeptide agents in both units. Colonization was detected in patients in traditionally lower-risk areas, such as medicine and surgery, as well as patients within the long-stay geriatric hospitals over the 30-month period.

Carriage and infection with VanB *E. faecium* occurred at a relatively constant level throughout the 30-month period (Fig. 2). Although only a selection of VanB *E. faecium* isolates were typed in this study, it appears that a dominant clone is present in a number of hospital units and patient groups in Glasgow. In contrast, isolation of VanA and VanC GRE was sporadic. PFGE analysis suggests these isolates were distinct (except for one cluster of VanA *E. faecium* isolates for which nosocomial spread was indicated).

The finding of a single *vanB E. faecium* clone that disseminated within and between hospitals parallels that seen in other studies (17, 22). The free movement of patients between the two major hospital units in this study may well have contributed to the dissemination of the clone in this instance. Furthermore, medical staff rotated between both sites, and although nursing staff usually worked on only a single ward, many worked extra shifts at other sites. Patients within the long-stay geriatric hospitals had often been previously admitted to either of the major hospitals.

The introduction of a fecal screening program greatly increased the rate of detection of GRE among inpatients. Thirty-six of 154 isolates originated from nonfecal sources and would have been detected by routine methods, as all were ampicillin resistant, necessitating testing for glycopeptide resistance. The isolates from the remaining 118 colonized patients, however, would not otherwise have manifested themselves, as during the study period none of the patients yielded GRE from nonfecal routine specimens. Detection of colonized patients allowed their isolation and reduced their risk as a source of infection. However, steady acquisition continued to occur despite these precautions. As detection of GRE outside the ICU relied on clinical staff deciding to send fecal specimens for other reasons, detection rates are probably far from complete and many patients colonized with GRE but in whom GRE were not detected are likely to have remained on the open ward.

The HICPAC-style fecal screening program did demonstrate a far greater extent of colonization than that which would have been revealed by more selective programs. However, colonization continued to occur, despite the knowledge obtained, and resources may have been better directed toward improving overall infection control and hand washing, as any patient, even in traditionally low-risk areas, may be potentially colonized with GRE. A point prevalence study in which every patient in the hospital on a given day is screened for the presence of GRE would give useful information on colonization and potential empirical treatment with considerable savings in resources.

Data collected from this program have given us an indication

of the background level of GRE at the study hospital trust, and this information will be of benefit should an outbreak of clinically significant GRE occur.

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# Tn*1546* Heterogeneity in VanA Enterococci from Scotland: Correlation between Genotype and Teicoplanin Resistance.

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**Background:** The genetic determinants responsible for conferring VanA-type glycopeptide resistance in enterococci frequently vary from the prototype Tn*1546* element described in *Enterococcus faecium* BM4147. Often these differences are attributable to insertion or deletion events upstream of *vanR* or downstream of *vanX*. In this study, the Tn*1546* elements of 48 VanA enterococcal isolates were studied for such variation.

**Methods:** Isolates were obtained from Scottish hospitals and confirmed as VanA-type GRE by standard methods. Tn*1546*-specific PCR primers were designed to amplify the *orf1-vanR*, *vanS-vanH* and *vanX-vanZ* regions of Tn*1546*. PCR products were subjected to RFLP analysis and some products were subsequently sequenced to confirm insertions or deletions.

**Results:** No isolates showed any variation from the prototype Tn1546 element within the *vanS-vanH* and *vanYZ* regions. However, the 48 isolates could be grouped into three groups according to variation within the *orf2-vanR* and *vanX-vanY* intergenic regions of theTn*1546* elements. Fifteen of the 48 isolates have no variation from the prototype Tn*1546* sequence (in terms of insertions/deletions). All of the remaining isolates harbor two distinct insertions: IS*1542* immediately upstream of *vanR*, and IS*1216V*-like element within the *vanX-vanY* intergenic region. Twenty-seven of these isolates also have a 93-bp deletion at the point of IS*1216V*-like insertion. The 34 isolates harboring both IS elements have a significantly higher teicoplanin MIC ($MIC_{50}$ & $MIC_{90}$ >1024mg/L) than those isolates with the prototype Tn*1546* ($MIC_{50}$ 64mg/L; $MIC_{90}$ 128mg/L). Sequencing of the IS*1542*/*vanR* region reveals a strong promoter sequence upstream of *vanR* that is generated by the insertion of IS*1542*.

**Conclusions:** The lack of variation within the *vanS-vanH* intergenic region is consistent with other European studies. Although IS*1216V*-like elements and IS*1542* have frequently been reported within Tn*1546*, we believe this to be the first correlation made between the presence of such IS elements and an increase in teicoplanin resistance. The insertion of IS*1542* generates a strong promoter sequence upstream of *vanR*, and also separates this promoter from the recognised phospho-VanR binding site that is thought, ordinarily, to repress *vanR* expression. It is proposed that these factors result in enhanced induction of resistance and may have a role in the mechanism for elevated teicoplanin resistance.